MolecularLibraries
Pathways to Discovery

Probe Report

Title: Identification of small molecules that selectively inhibit fluconazole-resistant *Candida albicans* in the presence of fluconazole but not in its absence—Probe 2

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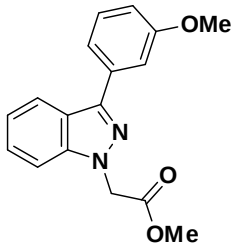
Assay Submitter & Institution: Susan Lindquist, PhD, Whitehead Institute, Cambridge, MA
PubChem Summary Bioassay Identifier (AID): 2007

Abstract:

The effectiveness of the potent antifungal drug fluconazole has been compromised by the rise of drug-resistant fungal pathogens. It has been observed that inhibition of Hsp90 can reverse drug resistance in *Candida*; however, it is challenging to find fungal-specific inhibitors of Hsp90 that do not also impair the human host protein. The MLPCN library was screened in duplicate dosings to identify compounds that selectively reverse fluconazole resistance in a *Candida albicans* clinical isolate, while having no antifungal activity when administered as a single agent. An indazole compound (CID3243873) was identified as meeting most of the probe criteria, and subsequent SAR identified a more potent analog as a new probe compound (ML212).

Probe Structure & Characteristics:



ML212

Compound Summary

IUPAC Chemical Name	methyl 2-(3-(3-methoxyphenyl)-1*H*-indazol-1-yl)acetate
PubChem CID	49835877
Molecular Weight	296.32
Molecular Formula	$C_{17}H_{16}N_2O_3$
ClogP	3.68
H-Bond Donor	0
H-Bond Acceptor	4
Rotatable Bond Count	5
Exact Mass	296.1161
Topological Polar Surface Area	51.13

CID/ML No.	Target Name	IC_{50} (nM) [SID, AID]	Anti-Target Names	IC_{50} (nM) [SID, AID]	Fold Selective*	Secondary Assay(s) IC_{50} (nM) [SID, AID]
49835877/ 212	CaCi-2 growth inhibition	440 [103910843, 493080]	Fibroblast toxicity	>26,000 [103910843, 493147]	59	CaCi-8 growth inhibition 1210 [103910843, 493149]
			Activity without fluconazole	>26,000 [103910843, 493070]	59	

*Selectivity = Anti-target IC_{50}/Target IC_{50}

Recommendations for the scientific use of this probe:

Acquired drug resistance by medically relevant microorganisms poses a grave threat to human health and has enormous economic consequences. Fungal pathogens present a particular challenge because they are eukaryotes and share many of the same mechanisms that support the growth and survival of the human host cells they infect. The number of drug classes that have unique targets in fungi is very limited, and the usefulness of current antifungal drugs is compromised by either dose-limiting host toxicity or the frequent emergence of high-grade resistance.

The objective of this project is to discover compounds capable of reversing fungal drug resistance, thereby making currently available antifungal drugs more effective and reducing dosage-related side effects. Inhibition of the molecular chaperone Hsp90 is one approach that can abrogate drug resistance in diverse fungi including species of both *Candida* and *Aspergillus* (1). At concentrations that demonstrate no antifungal activity on their own, classical Hsp90 inhibitors dramatically reduce azole antibiotic resistance of clinical isolates and transform conventional fungistatic azole compounds into fungicidal drug combinations. This probe could greatly improve the ability to control otherwise lethal fungal infections in severely immunocompromised hosts, especially cancer patients undergoing high-dose chemotherapy and/or bone marrow transplantation. This high-throughput screen (HTS) project seeks to identify novel small molecules that can prevent fungal growth in combination with a sublethal dose of fluconazole.

1 Introduction

Scientific Rationale

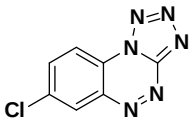
The molecular chaperone Hsp90 has been previously linked to the evolution of antifungal drug resistance in various fungal strains (1). In these studies, the inhibition of Hsp90 can abrogate drug resistance in diverse fungi, including species of both *Candida* and *Aspergillus*. At concentrations that demonstrate no antifungal activity on their own, classical Hsp90 inhibitors dramatically reduce azole antibiotic resistance of clinical isolates and transform conventional fungistatic azole compounds into fungicidal treatments (1).

The resistance phenotype mediated by a wide variety of specific mutations is contingent on intact Hsp90-dependent fungal stress responses (1). Such responses allow the organism to tolerate the stresses imposed by drug exposure itself and/or accommodate mutations that mediate drug resistance. For example, genetic compromise of the key fungal stress signaling molecule calcineurin, itself an Hsp90 client protein, faithfully phenocopies the effects of Hsp90 inhibition (1). Modulators of the fungal stress response could greatly improve the ability to control otherwise lethal fungal infections in immunocompromised hosts, especially cancer patients undergoing high-dose chemotherapy and/or bone marrow transplant procedures.

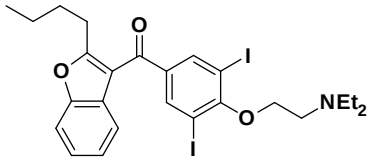
Although Hsp90 or calcineurin inhibitors under current development appear to be well-tolerated in early phase cancer clinical trials, compromise of host chaperone protein function could have very deleterious effects in the context of active infection and the associated stresses of fever and cardiovascular instability. For example, host immune response is compromised by treatment with nonselective calcineurin inhibitors such as cyclosporin A and FK506. An obvious way to avoid this problem would be the identification of fungal-specific inhibitors that do not impair the host chaperone protein. To address these concerns, this project seeks to identify new fungal-selective chemosensitizers. In particular, the design of the screening cascade will also allow capture of compounds targeting still other, as yet unknown, components of fungal stress response pathways that enable the emergence and maintenance of resistance to current antifungal drugs. Facilitating this strategy, secondary binning assays were included in the screening path and were designed to characterize the phenotypic hits by their species specificity (fungal *vs.* human cells) and by apparent mode of action (*Hsp90-related, calcineurin-related, or Unknown/Other mechanism*).

Several compounds have been previously identified as chemosensitizers, increasing the susceptibility of various *C.albicans* strains to fluconazole treatment (2,3,4,5). Cernicka et al. previously reported that the compound 7-chlorotetrazolo[5,1-*c*]benzo[1,2,4]triazine (CTBT, **Figure 1**) was capable of chemosensitizing *C.albicans* strains to fluconazole (3). Against fluconazole-susceptible *C.albicans* strain 90028 and fluconazole-resistant *C.albicans* strain Gu5, CTBT was effective with an MIC value of 2.4 µM when combined with fluconazole. In the absence of fluconazole, CTBT demonstrated no activity against *C.albicans* strain 90028, but did inhibit growth of *C.albicans* strain Gu5 at concentrations greater than 2.4 µM. The anti-arrhythmic drug amiodarone was recently demonstrated to act synergistically with fluconazole in *C.albicans* with MIC values ranging between 1.6 µM to 18.8 µM (4). Plagiochin E, a natural product isolated from liverwort, increased yeast susceptibility to fluconazole at 2.4 µM (5). These agents were not considered for the current project because of their documented single-agent antifungal activity (amiodarone an MIC_{50} of 3.1 µM [6]); plagiochin E an IC_{50} value of 3.8 µM [5]).

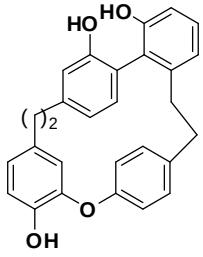
Figure 1. Chemosensitizing Agents for Reversing Fluconazole Resistance



CTBT (1)



Amiodarone (2)

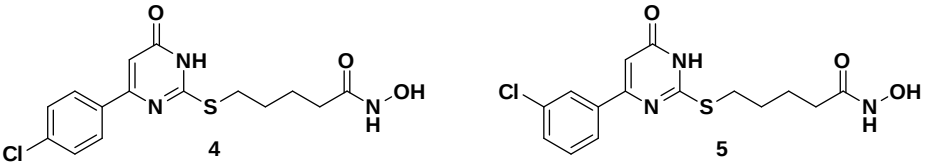


Plagiochin E (3)

The most potent compounds currently known are several HDAC inhibitors previously reported by Mai et al. (7). As depicted in **Figure 2**, compounds **4** and **5** are uracil-derived hydroxamic acids that exhibited MIC values ranging from 1.2 µM to 1.4 µM when combined with fluconazole. When tested independently, neither compound demonstrated activity against *C. albicans* at concentrations up to 368 µM. When compounds **4** and **5** were evaluated in a biochemical binding assay with murine HDAC1, their IC_{50} values were measured at 37 nM and 51 nM, respectively (7). In addition, closely related analogs inhibit human HDAC1 and HDAC4 and were further shown to possess antiproliferative and cytotoxic effects against several human cell lines (8,9). These findings strongly suggest that compounds **4** and **5** would not be particularly selective for fungal protein targets, diminishing their potential as fungal-selective chemosensitizers. At the present time, compounds **4** and **5** have not been registered with MLSMR, are not sold commercially, and as such, are not available for evaluation in the current investigation. Ideally, screening the MLPCN compound collection would yield novel chemical

hits that work by new modes of action that could be investigated by the Assay Provider, but one back-up option that is available is to identify fungal selective HDAC inhibitors.

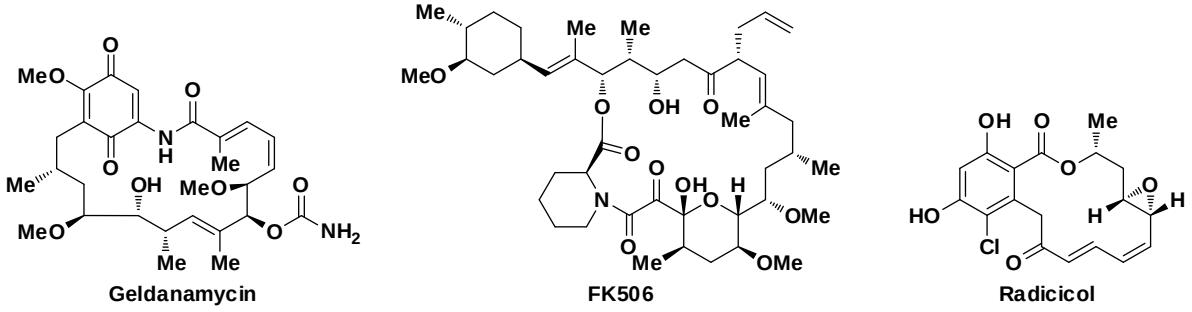
Figure 2. Uracil-derived HDAC Inhibitors Capable of Reversing Antifungal Drug Resistance



1 Materials and Methods

Compounds that can successfully inhibit *Candida* CaCi-2 cell growth in the presence of sublethal doses of fluconazole as measured in a fluorescence reporter assay were further tested against a highly-resistant *Candida* CaCi-8 strain in the presence of fluconazole. Those that inhibited growth in the resistant strain were also tested for toxicity against *Candida* in the absence of fluconazole and for toxicity against mammalian cells using a fluorescence reporter assay. The positive control for screening in the *Candida* and mammalian cell assays was the Hsp90 inhibitor geldanamycin (acting in concert with fluconazole; see **Figure 3**). Compounds that passed these four hurdles were binned through use of a *Saccharomyces* assay to determine whether the mechanism of action might be through the Hsp90 pathway, the calcineurin pathway, or an uncategorized mechanism.

Figure 3. Positive Controls for Biological Assays



2.1 Assays

A summary listing of completed assays and corresponding PubChem AID numbers is provided in **Appendix A** (Table A1). Refer to **Appendix B** for the detailed assay protocols.

2.1.1 Primary CaCi-2 (AID No. 1979), CaCi-2 Dose-Response Retest (AID Nos. 2467, 488836)

Materials and Reagents:

Clear, flat-bottom, black, 384-well plates were obtained from Corning (Catalog no. 3712BC, Lot no. 35808016). Geldanamycin (Catalog no. G-1047) was obtained from AG Scientific and prepared in 15 mM stock solution in DMSO (control). Fluconazole was obtained from Sequoia Research Products Ltd. and prepared in a 2 mg/ml stock solution in phosphate buffered saline (PBS). Alamar Blue was obtained from Biosource International (Catalog no. DAL1100, Lot no. 151016SA). PBS (Catalog no. 21-040-CV) without calcium and magnesium was obtained from Cellgro. Penicillin/Streptomycin (Pen/Strep) was obtained from Gibco and prepared 100X in PBS.

Synthetic Defined Growth Medium

RPMI 1640 medium, (powder without sodium bicarbonate; Catalog no. 31800-089, Lot no. 648072) was obtained from Invitrogen. Uridine 8 mg/ml in water (Catalog no.U3750, Lot no. 028KO760), glucose 40% (w/v) in water (Catalog no. G-5400), and MOPS Buffer (Catalog no. M-1254, Lot no. 098K0033) were obtained from Sigma.

RPMI medium (1X) was prepared by dissolving 10.4 g powdered medium in 800 ml water. A buffer of 34.52 g MOPS was added. While stirring, pH was adjusted to 7.0 with 10N NaOH. Next, 10 ml uridine solution and 50 ml glucose solution were added. The final volume was adjusted to 1000 ml, and filter sterilized.

Fungal Inoculum

The following yeast strains were used in this study: *C. albicans* CaCi-2. Fungal inoculum was prepared as follows: 500 µl of strain was inoculated from cryopreserved stock into a 250-ml shaker flask containing 30 ml growth medium and shaken overnight at 30 °C.

The optical density (OD 600) of 1 ml fungal culture in a cuvette was read using a standard optical density reader (Eppendorf BioPhotometer Plus), with growth medium as a background blank. The desired volume of fungal inoculums was diluted according to the formula specified in the protocol (see **Appendix B**).

Procedures:

Fluconazole stock solution was added to the fungal inoculum to achieve 8 µg/ml. Pen/Strep was added to the media to a 1% concentration (v/v). A Thermo Combi nL was used to dispense 20 µl/well of

assay media into all wells. Geldanamycin was dispensed in positive control wells using Thermo Combi nL for a final concentration of 3 µM. Then, 100 nl of test compound was pinned from compound plates into assay plates using a CyBi-Well pin tool. A further 20 µl/well of culture was dispensed into the assay media in all wells. The plates were incubated in a humidified (90 % humidity) Liconic incubator at 37 °C without agitation for 48 hours. Alamar Blue was diluted 1:40 in Ca/Mg-free PBS. To all plates, 5 µl/well of the diluted Alamar Blue was added for a final dilution factor of 1:200. The plates were incubated for an additional 2 hours. Then, Relative Fluorescence Intensity (RFU) of each well was read on standard plate reader as a measure of relative fungal growth. Envision (Perkin Elmer) plate reader set-up: Ex 544 nm, Em 590 nm, Bandwidth 12 nm, Top read.

2.1.2 Counterscreen Mammalian Cell Toxicity Assay (AID Nos. 2327, 488809, 493099, 493147)

Materials and Reagents:

Clear, flat-bottom, black, 384-well plates were obtained from Corning (Catalog no. 3712BC, Lot no. 35808016). Geldanamycin (Catalog no. G-1047) was obtained from AG Scientific and prepared in 15 mM stock solution in DMSO (control). Fluconazole was obtained from Sequoia Research Products Ltd. and prepared in a 2 mg/ml stock solution in PBS. Alamar Blue was obtained from Biosource International (Catalog no. DAL1100, Lot no. 151016SA). Phosphate buffered saline (PBS; Catalog no. 21-040-CV) without calcium and magnesium was obtained from Cellgro.

Assay Medium

Optimem medium (Catalog no.31985-070, Lot no. 548536) and Pen/Strep 1% (v/v) solution (Catalog no.15140-122, Lot no. 529891) were obtained from Invitrogen. Fetal bovine serum 2.5% (v/v) (FBS; Catalog no. 30071.03, Lot no. ARF26748) was obtained from Hyclone.

Cell Inoculum

The following test strain was used in this study: NIH-3T3 mammalian fibroblasts (ATCC; CRL no.1658). Cell inoculum was prepared as follows: Cells were plated at 6,000 cells/well in 20 µl assay medium and cultured overnight at 37 °C under 5% CO_2 in 384-well, clear bottom, black, tissue culture-treated, bar-coded assay plates.

Procedures:

After overnight culture, compounds were pinned into wells at 100 nl/well using the CyBio CyBi-Well pinning instrument. After compounds were pinned, an additional 20 µl of assay medium supplemented with fluconazole (16 µg/ml) was added to each well. The final nominal concentration in the well was 50 µM of test compound and 8 µg/ml fluconazole. The plates were returned to the tissue culture incubator, and the culture continued for an additional 48 hours at 37 °C under 5% CO_2. At the completion of this incubation, Alamar Blue Reagent diluted 1:40 in Ca/Mg-free PBS was added to each well (10 µl/well) to achieve a final dilution of 1:200. The plates were incubated for an additional 2 to 3 hours at 37 °C under 5% CO_2, and then RFU as a measure of relative viable cell number was determined on an EnVision plate reading fluorometer. EnVision (Perkin Elmer) plate reader set-up: Ex 544 nm, Em 590 nm, Bandwidth 12 nm, Top read.

2.1.3 Secondary Single-Agent (No-Fluconazole) Activity Assay (AID No. 488802)

Materials and Reagents:

Clear, flat-bottom, black, 384-well plates were obtained from Corning. Geldanamycin was obtained from AG Scientific G-1047 and prepared in 15 mM stock solution in DMSO. Pen/Strep was obtained from Gibco and prepared 100X in PBS. Fluconazole was obtained from Sigma and prepared in 2 mg/ml stock solution in PBS. Alamar Blue was obtained from Biosource International (Catalog no. DAL1100). Phosphate buffered solution (PBS) without calcium and magnesium was obtained from Cellgro.

Synthetic Defined Growth Medium

For the synthetic defined growth medium, RPMI 1640 medium (powder without sodium bicarbonate) was obtained from Invitrogen. Uridine (8 mg/ml in water), glucose 40% (w/v) in water, and MOPS buffer was obtained from Sigma.

RPMI medium (1X) was prepared by dissolving 10.4 g powdered medium in 800 ml water. A buffer of 34.52 g MOPS was added. While stirring, pH was adjusted to 7.0 with 10N NaOH. Next, 10 ml uridine solution and 50 ml glucose solution were added. The final volume was adjusted to 1000 ml, and the solution was filter sterilized.

Fungal Inoculum

The following yeast strain was used in this study: *C. albicans* CaCi-2 (10). Fungal inoculum was prepared as follows: 500 µl of yeast was inoculated from cryopreserved stock into a 250-ml shaker flask containing 30 ml growth medium and shaken overnight (16 hours) at 30 °C. The culture was spun down, and the broth was poured off and washed with RPMI medium. The culture was spun down again, and the broth was poured off and resuspended in RPMI medium. The OD 600 of 1 ml of fungal culture in a cuvette was read using a standard optical density reader with growth medium as a background blank. The desired volume of fungal inoculum was diluted to starting OD of the fungal inoculum of 0.00015 A600.

Procedures:

Pen/Strep was added to the media to a final 1% concentration. A Combi NL (Thermo) was used to dispense 20 µl/well of assay media into all wells. Geldanamycin (1.5 mM) and fluconazole (0.2 mg/ml) were mixed for the positive control. Then, 80 nl of positive control solution was dispensed into the positive control wells using Thermo Combi nL for a final concentration of 3 µM geldanamycin and 8 µg/ml fluconazole. Then, 100 nl of test compound were pinned from the compound plates into assay plates using a CyBi-Well pin tool. A further 20 µl/well of RPMI synthetic defined medium culture was dispensed into 384-well, black plates. Fungal suspension (20 µl/well) was dispensed into all wells. The plates were incubated in a humidified (90% humidity) Liconic incubator at 37 °C without agitation for 48 hours. Alamar Blue Reagent was diluted 1:40 in Ca/Mg-free PBS. To all plates, 6.4 µl/well of the diluted Alamar Blue was added for a final dilution factor of 1:200. The plates were incubated for 2 hours at room temperature. Next, RFU of wells was read on a standard plate reader as a measure of relative fungal growth. Envision (Perkin Elmer) plate reader set-up: Ex 544 nm, Em 590 nm, Bandwidth 12 nm, Top read.

2.1.4 Hsp90 Binning Materials and Reagents: Corning white, 384-well plates were obtained from Corning (Catalog no. 8867BC; Lot no. 22609019). Tropix Gal-Screen was obtained from Applied Biosystems (Catalog no. T2359; Lot no. 0903044).

Assay Media

SD-ADE yeast nitrogen base w/o ammonium sulfate, minus adenine

SD Growth Media was obtained from MP Biomedical (Catalog no. 4027-012; Lot no. 119458). Complete Supplement minus adenine was obtained from Sunrise Science (Catalog no.1029-100; Lot no. 070409). Deoxycorticosterone (DOC) steroid was obtained from Sigma.

To prepare the assay media, 100 ml (20% (w/v) dextrose and 780 mg Complete Supplement was added to 100 ml SD Growth Media. Water was added to a final volume of 1 liter, and the solution was filter sterilized.

DOC Media

For DOC media, 1 ml DOC was added to 100 ml SD-ADE media.

Cell Inoculum

The following test strain was used in this study: *Saccharomyces cerevisiae* W303 reporter strain (ATCC 201238). Cell Inoculum was prepared as follows: Reporter *Saccharomyces* strain was inoculated from cryopreserved stock into a 250 ml shaker flask containing 20 ml SD-ADE media. The reporter strain W303 was incubated overnight (16 hours) at 37 °C and 150 rpm.

Procedures:

The OD 600 of 1 ml of culture in a cuvette was read using a standard optical density reader with growth medium as a background blank. Cells were diluted to OD = 0.04 in SD-ADE media. To each 384-well white plate, 20 µl of diluted culture was dispensed using a Combi NL (Thermo). Then, 100 nl of test compound were pinned into plates with a CyBi-Well pin tool. Next, 5 µM radicicol was added as positive control in the control wells, dispensing with a Combi NL (Thermo). With Combi, 20 µl of 20 µM DOC (steroid) in SD-ADE media was dispensed in pinned plates. The plates were incubated at 30 °C for 75 minutes with agitation. Using Combi, 40 µl Gal-Screen reagent was dispensed. The plates were incubated at 30 °C for 25 minutes. Luminescence of the wells was read on a standard plate reader as a measure of relative fungal growth. Envision (Perkin Elmer) plate reader set-up**:** Top read; Luminescence filter (560 nm) at 0.1 seconds.

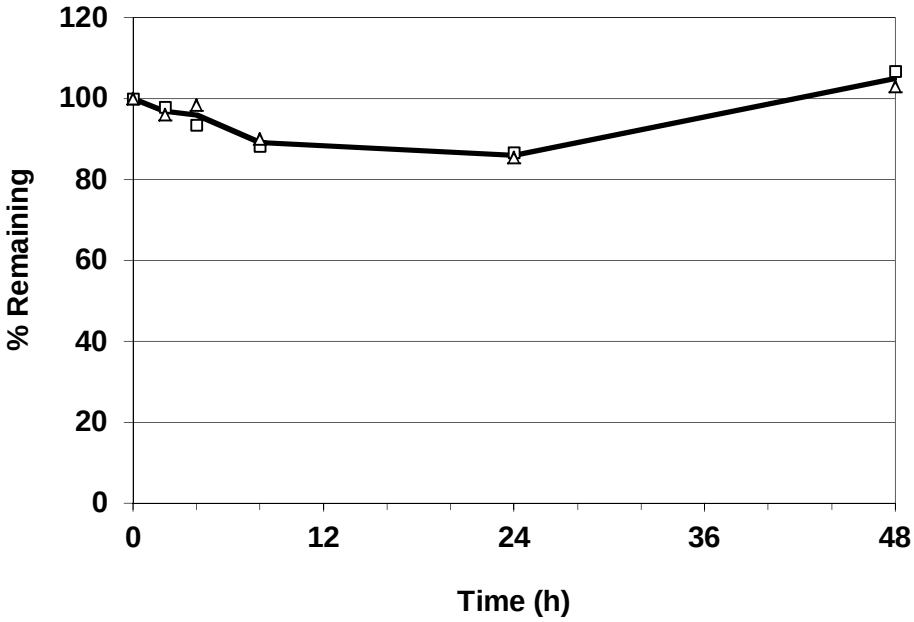
2.2 Probe Chemical Characterization

After preparation as described in Section 2.3, the probe (ML212) was analyzed by UPLC, ^{1}H and ^{13}C NMR spectroscopy, and high-resolution mass spectrometry. The data obtained from NMR and mass spectroscopy were consistent with the structure of the probe, and UPLC indicated an isolated purity of greater than 93%. The relevant data is provided in **Appendix C**.

The solubility of the probe (ML212) was experimentally determined to be 3.6 µM in 2% (v/v) DMSO/PBS solution. The probe is exceptionally stable in PBS solution (>99% remaining after a 48-hour incubation). The data from the PBS stability assay is provided in **Figure 4**. Plasma protein binding (PPB) was determined to be 95% bound in human plasma. The probe is unstable in human plasma with approximately 3% remaining after a 5-hour incubation period. Presumably, hydrolysis of the methyl ester is the primary contributor to instability. The solubility, PPB, and plasma stability results are summarized in Section 3.4 (entry 8, **Table 5**).

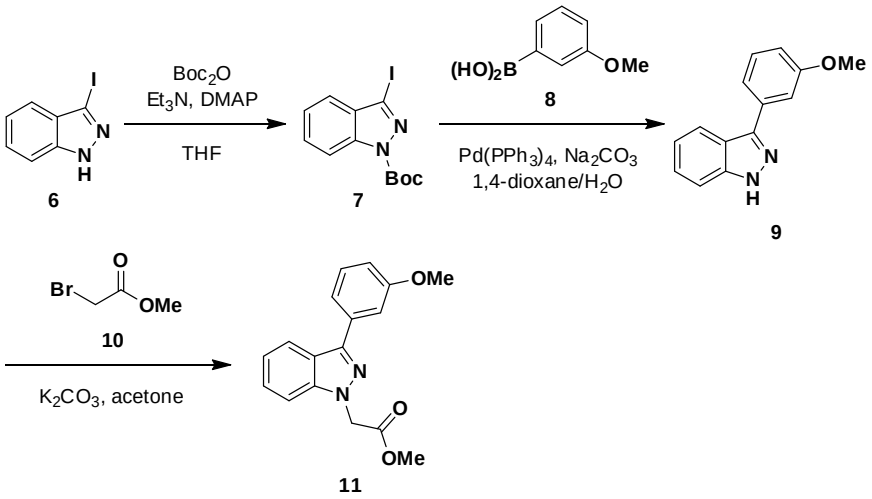
The probe (ML212) and five additional analogs were submitted to the SMR collection MLS003271341 (probe), MLS003271339 (CID100493), MLS003271340 (CID49835836), MLS003271342 (CID49835857), MLS003271343 (CID3243873), and MLS003271344 (CID49835842).

Figure 4. Stability of the Probe (ML212) in PBS at 23 °C



2.3 Probe Preparation

Scheme 1. Synthesis of the Probe (ML212)

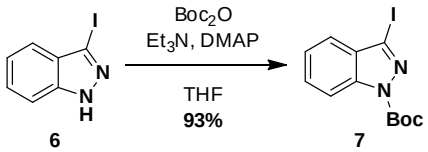


The probe compound **11** (ML212) was prepared in a three-step sequence beginning with commercially available 3-iodo-1*H*-indazole (see **Scheme 1**). This material was first protected as its *tert*-butyl carbamate then coupled with 3-methoxyphenyl boronic acid. Conveniently, the thermal, alkaline conditions of the Suzuki coupling facilitated *in situ* decomposition of the Boc protecting group (11-13), and coupling product was isolated as only the free indazole. Subsequent alkylation with methyl

bromoacetate completed the synthesis of the probe. Full experimental details are provided in this section.

General details. All reagents and solvents were purchased from commercial vendors and used as received. NMR spectra were recorded on a Bruker 300 MHz spectrometer. Proton and carbon chemical shifts are reported in ppm (δ) relative to tetramethylsilane (^{1}H δ 0.00) or residual chloroform in $CDCl_3$ solvent (^{1}H δ 7.24, ^{13}C δ 77.0). NMR data are reported as follows: chemical shifts, multiplicity (obs. = obscured, br = broad, s = singlet, d = doublet, t = triplet, m = multiplet); coupling constant(s) in Hz; integration.

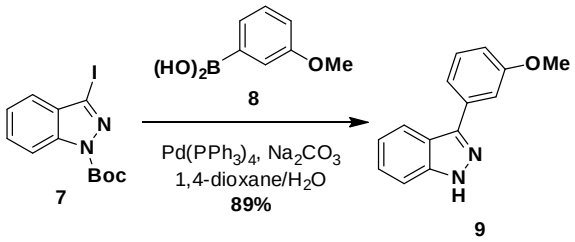
Unless otherwise indicated, NMR data were collected at 25 °C. Flash chromatography was performed using 40-60 µm Silica Gel (60 Å mesh) on a Teledyne Isco Combiflash R_f system. Tandem Liquid Chromatography/Mass Spectrometry (LC/MS) was performed on a Waters 2795 separations module and 3100 mass detector. Analytical thin layer chromatography (TLC) was performed on EM Reagent 0.25 mm silica gel 60-F plates. Visualization was accomplished with UV light and aqueous potassium permanganate ($KMnO_4$) stain followed by heating. Microwave reactions were performed with a Biotage Initiator 2.5 Microwave Synthesizer. High-resolution mass spectra were obtained at the MIT Mass Spectrometry Facility with a Bruker Daltonics APEXIV 4.7 Tesla Fourier Transform Ion Cyclotron Resonance mass spectrometer. Compound purity and identity were determined by UPLC-MS (Waters, Milford, MA). Purity was measured by UV absorbance at 210 nm. Identity was determined on an SQ mass spectrometer by positive electrospray ionization. Mobile Phase A consisted of either 0.1% ammonium hydroxide or 0.1% trifluoroacetic acid in water, while mobile Phase B consisted of the same additives in acetonitrile. The gradient ran from 5% to 95% mobile Phase B over 48 seconds at 0.45 ml/min. An Acquity BEH C18, 1.7 µm, 1.0 x 50 mm column was used with column temperature maintained at 65 °C. Compounds were dissolved in DMSO at a nominal concentration of 1 mg/ml, and 0.25 µl of this solution was injected.



Step 1. Preparation of *tert*-butyl 3-iodo-1*H*-indazole-1-carboxylate (7): 3-Iodo-1*H*-indazole (5.00 g, 19.5 mmol) was placed in a round-bottom flask and dissolved in tetrahydrofuran (100 ml). 4-Dimethylaminopyridine (0.24 g, 1.9 mmol, 0.1 equiv) was then added, followed by di-*tert*-butyl

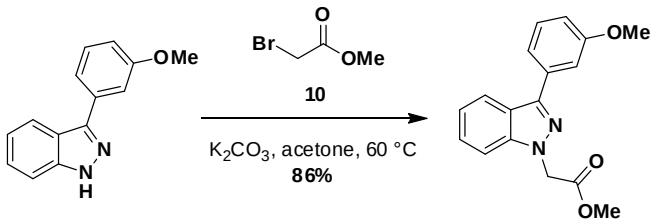
dicarbonate (5.4 ml, 24 mmol, 1.2 equiv). Triethylamine (5.4 ml, 39 mmol, 2.0 equiv) was slowly added to the clear brown solution by syringe. The resulting solution was stirred at room temperature and monitored by TLC until complete. The reaction required approximately 2 hours. Once complete, the reaction was diluted with water (75 ml) and ethyl acetate (50 ml). After separating the layers, the aqueous phase was extracted with additional ethyl acetate (3 x 50 ml). The combined organic layers were washed with brine (100 ml), then shaken over magnesium sulfate, filtered, and concentrated under reduced pressure to give a dark red oil (8.40 g). The crude material was purified by column chromatography over silica gel (hexanes/ethyl acetate: 100/0 to 90/10) to give the title compound as an orange solid (6.20 g, 93%).

^{1}H NMR (300 MHz, $CDCl_3$): δ 8.12 (d, *J* = 8.4 Hz, 1H), 7.59 (t, *J* = 7.7 Hz, 1H), 7.50 (d, *J* = 7.9 Hz, 1H), 7.37 (t, *J* = 7.5 Hz, 1H), 1.73 (s, 9H); **^{13}C NMR (75 MHz, $CDCl_3$):** δ 148.3, 139.6, 130.2, 129.9, 124.1, 121.9, 114.5, 102.8, 85.4, 28.1; **ESI-MS (M-C_4H_9):** *m/z* 288.



Step 2. Preparation of (3-(3-methoxyphenyl)-1*H*-indazole (9): *tert*-butyl 3-iodo-1*H*-indazole-1-carboxylate (100 mg, 0.29 mmol) was placed in a suitably sized microwave vial and dissolved in 1,4-dioxane (11.5 ml). 3-Methoxyphenyl boronic acid (88 mg, 0.58 mmol, 2.0 equiv) and tetrakis(triphenylphosphine) palladium (20 mg, 0.017 mmol, 0.06 equiv) were added, and the resulting turbid orange mixture was sparged thoroughly with nitrogen**.** An aqueous solution of sodium carbonate (2.0 M, 0.65 ml, 1.31 mmol, 4.5 equiv) was then added. The biphasic mixture was microwaved for 1 hour at a reaction temperature of 120 °C. The reaction was diluted with ethyl acetate (2 ml), and then filtered through a celite pad with additional ethyl acetate. The filtrate was concentrated under reduced pressure to give an oil. The crude material was purified by column chromatography over silica gel (hexanes/ethyl acetate: 100/0 to 30/70) to give the title compound as an orange oil (58.0 mg, 89%).

^{1}H NMR (300 MHz, $CDCl_3$): δ 12.71 (s, 1H), 8.00 (d, *J* = 8.2 Hz, 1H), 7.69 – 7.53 (m, 2H), 7.44 (t, *J* = 7.9 Hz, 1H), 7.31 – 7.22 (m, 1H), 7.17 (dd, *J* = 4.9, 13.0 Hz, 1H), 7.05 (d, *J* = 8.3 Hz, 1H), 7.03 – 6.98 (m, 1H), 3.79 (s, 3H); **^{13}C NMR (75 MHz, $CDCl_3$):** δ 160.1, 145.4, 141.7, 134.8, 130.0, 126.7, 121.3, 120.9, 120.8, 120.3, 114.2, 113.0, 110.5, 55.3; **ESI-MS:** *m/z* 224



Step 3. Preparation of methyl 2-(3-(3-methoxyphenyl)-1*H*-indazol-1-yl)acetate (11): 3-(3-methoxyphenyl)-1*H*-indazole (163 mg, 0.73 mmol) was dissolved in acetone (2.2 ml) and treated with methyl bromoacetate (0.21 ml, 2.2 mmol, 3.0 equiv). Finely powdered potassium carbonate (721 mg, 2.2 mmol, 3.0 equiv) was added in a single portion, and the resulting suspension was stirred overnight at 60 °C. Upon completion, the reaction was cooled to room temperature and filtered through celite with acetone. The clear orange filtrate was concentrated under reduced pressure to give the crude product as a dark orange oil. This material was purified by column chromatography over silica gel (hexanes/ethyl acetate: 100/0 to 70/30) to give the probe compound as an orange oil (186 mg, 86%).

^{1}H NMR (300 MHz, $CDCl_3$): δ 8.03 (d, *J* = 8.2 Hz, 1H), 7.53 (obs. t, *J* = 6.0 Hz, 1H), 7.50 (obs. s, 1H), 7.42 (obs. q, *J* = 7.7 Hz, 2H), 7.36 (obs. t, *J* = 8.1 Hz, 1H), 7.24 (t, *J* = 7.5 Hz, 1H), 6.96 (dd, *J* = 2.5, 8.2 Hz, 1H), 5.22 (s, 2H), 3.89 (s, 3H), 3.75 (s, 3H). **^{13}C NMR (75 MHz, $CDCl_3$):** δ 168.4, 160.0, 145.1, 141.7, 134.6, 129.8, 126.9, 122.2, 121.6, 121.5, 120.1, 114.2, 112.8, 109.0, 55.4, 52.6, 50.3. **HRMS (ESI):** calculated mass for $C_{17}H_{16}N_2O_3$ [M+H] 297.1234, found 297.1246.

2.4 Additional Analytical Analysis

Plasma Protein Binding. Plasma protein binding was determined by equilibrium dialysis using the Rapid Equilibrium Dialysis (RED) device (Pierce Biotechnology, Rockford, IL) for both human and mouse plasma. Each compound was prepared in duplicate at 5 µM in plasma (0.95% acetonitrile, 0.05% DMSO) and added to one side of the membrane (200 µl) with PBS pH 7.4 added to the other side (350 µL). Compounds were incubated at 37 °C for 5 hours in a 250-rpm orbital shaker. Following the incubation, the samples were analyzed by UPLC-MS (Waters, Milford, MA) with compounds detected by SIR detection on a single quadrupole mass spectrometer.

Plasma Stability. Plasma stability was determined at 37 °C for 5 hours in both human and mouse plasma. Each compound was prepared in duplicate at 5 µM in plasma diluted 50/50 (v/v) with PBS pH 7.4 (0.95% acetonitrile, 0.05% DMSO). The compounds were incubated at 37 °C for 5 hours with a 250-rpm orbital shaker with time points taken at 0 hours and 5 hours. The samples were analyzed by UPLC-MS (Waters, Milford, MA) with compounds detected by SIR detection on a single quadrupole mass spectrometer.

3 Results

Probe attributes:

- Compounds that inhibit yeast growth in the presence, but not in the absence of 8 µg/ml fluconazole.
- Compounds that show at least 10-fold selectivity between the primary *Candida* test strain and mammalian cells.
- Compounds that show activity toward resistant clinical isolates at an IC_{50} < 50 µM.
- $IC_{50} \leq 1$ µM in primary or resistant screen cell line.

3.1 Summary of Screening Results

Figure 6 displays the critical path for probe development.

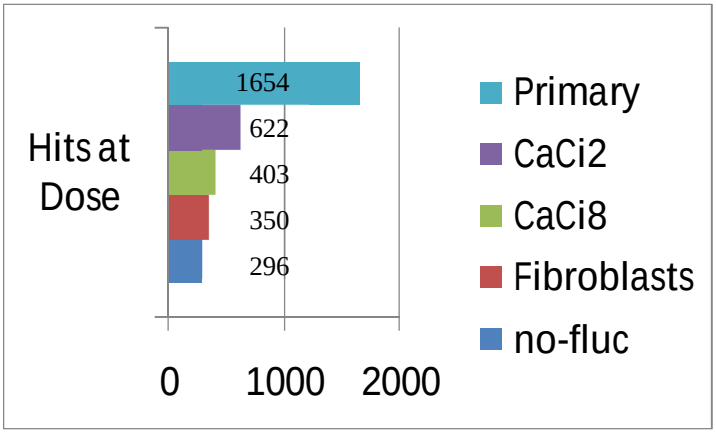
A high-throughput screen of 302,509 compounds (PubChem AID 1979) was performed in duplicate in the fluconazole-resistant *C. albicans* strain CaCi-2 in the presence of a sublethal concentration of fluconazole. Using a screening active cutoff of ≥75% inhibition at a screening concentration of 9.5 µM, 1893 hits were identified as *Candida* CaCi-2 growth inhibitors in the presence of fluconazole, and 1654 were available as cherry picks. These picked compounds were retested in dose against the *C. albicans* strain CaCi-2 to confirm their inhibitory activity and determine an IC_{50} value. Of these, there were 622 compounds that met the criterion of inhibitory activity of less than or equal to 1 µM.

An orthogonal screen of these 1654 cherry picks against a more resistant *C. albicans* strain, CaCi-8 in the presence of a sub-lethal concentration of fluconazole (AID 2423), selected for compounds that were active with an IC_{50} value less than 50 µM. There were 836 compounds that met this criterion, and 403 of these compounds were among the 622 actives from the CaCi-2 retest.

Murine 3T3 fibroblasts provided an assay for overt compound toxicity to mammalian cells. Of the 1654 cherry picks, 1012 compounds were inactive in this assay, indicating fungal selectivity, of which 350 also met the criteria in the prior CaCi-2 and CaCi-8 assays.

To eliminate cherry pick compounds that intrinsically inhibit *Candida* growth, an additional secondary screen of the 1654 cherry picks in the absence of fluconazole was included, with a 10 µM IC_{50} cutoff. Of the 350 compounds of interest, 296 compounds met this criterion.

Figure 5. Bar Chart of the Antifungal Screening Campaign



Two ancillary secondary assays were run to bin the remaining 296 compounds into three classes: Hsp90 inhibitors, calcineurin inhibitors, or other. Inhibition of either Hsp90 or calcineurin has been shown to restore fluconazole sensitivity in *Saccharomyces cerevisiae* as well as *Candida albicans,* but the specific mechanism(s) by which resistance is lost remains unknown. Identification of fungal-selective Hsp90 or calcineurin inhibitors would enable more detailed investigation into how Hsp90 engenders antifungal drug resistance in fungi.

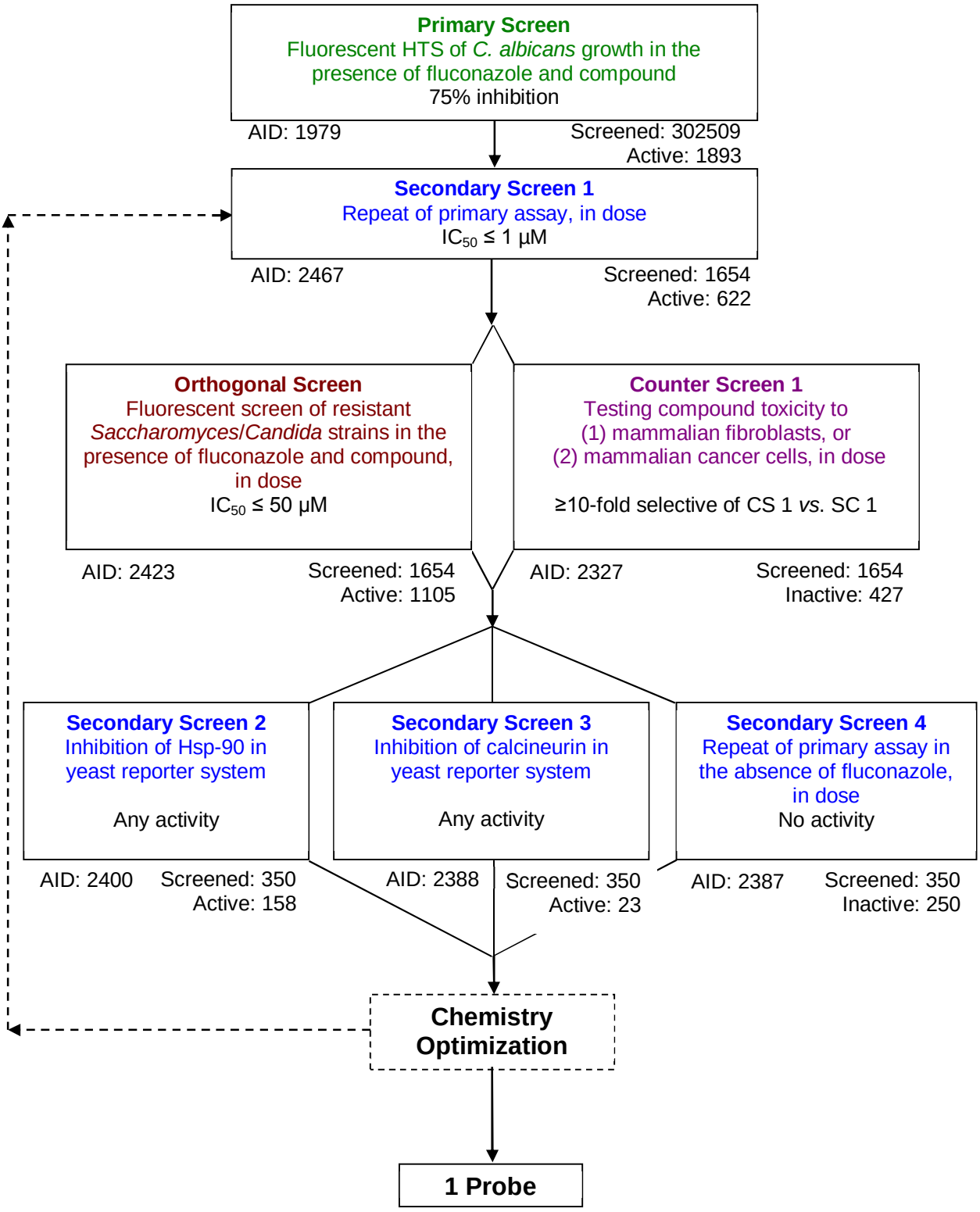
The Hsp90 assay used a *Saccharomyces cerevisiae* strain engineered to express beta-galactosidase driven by glucocorticoid response element. The glucocorticoid hormone receptor depends heavily on Hsp90 for function. Of the 296 compounds of interest, 17 compounds were active as defined by a 10 µM upper threshold of inhibition.

The second binning assay for calcineurin inhibition was evaluated in a yeast carrying a construct encoding calcineurin-dependent response elements (CDRE) driving expression of beta-galactosidase. Reporter activity with or without the prior addition of test compounds was measured following challenge with the stressor calcium chloride ($CaCl_2$). Of the 296 compounds of interest, two compounds were

active as defined by a 10 µM upper threshold of inhibition. The remaining 277 compounds functioned *via* alternative pathways and were categorized as “Other/Unknown mechanism.”

Thirty (30) compounds were chosen for initial dry powder confirmation studies from the 296 identified above by first clustering into small groups of related analogs, and then picking representative analogs from each of those families. After re-testing these dry powders in the test cascade, three compounds were chosen as potential probe candidates, and a first round of 31 analogs (plus three probe candidates) were obtained and assayed. Using the results from these assays as guidance, a second round of analogs (plus the probe candidate) was prepared for SAR analysis.

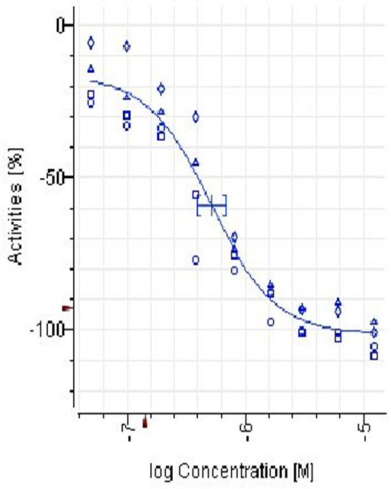
Figure 6. Critical Path for Probe Development



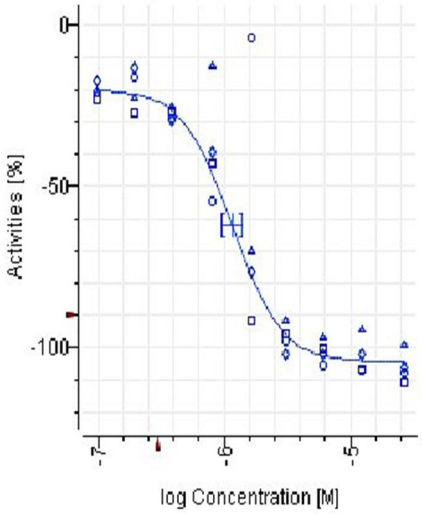
3.2 Dose Response Curves for Probe

Figure 7. Dose-dependent Activity of the Probe (ML212) Against Various Cell Lines. *C.albicans* CaCi-2 in the presence of fluconazole (IC_{50} = 440 nM, AID 493080) (A); *C.albicans* CaCi-8 in the presence of fluconazole (IC_{50} = 1210 nM, AID 493149) (B); Murine 3T3 fibroblasts in the absence of fluconazole (inactive, AID 493147) (C); *C.albicans* CaCi-2 in the absence of fluconazole (inactive, AID 493070) (D); *S. cerevisiae* with Hsp90 construct (IC_{50} = 4.18 µM, AID 493134) (E).

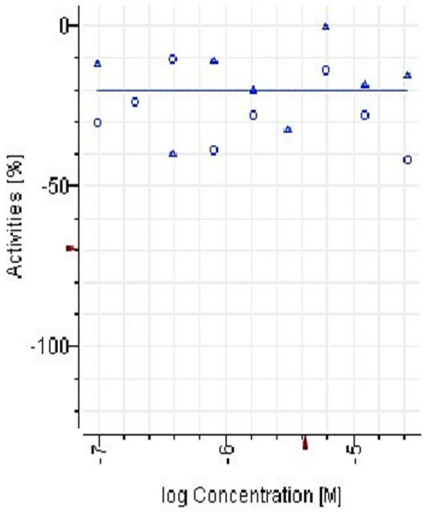
A.



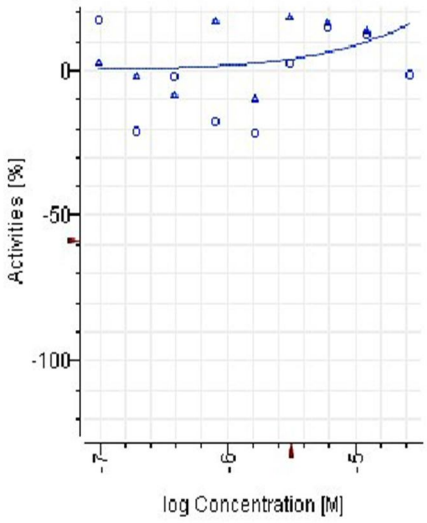
B.



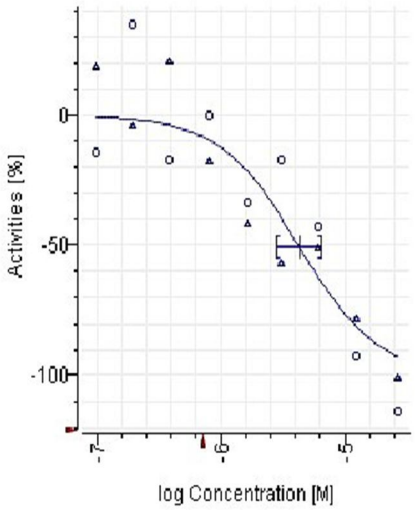
C.



D.



E.



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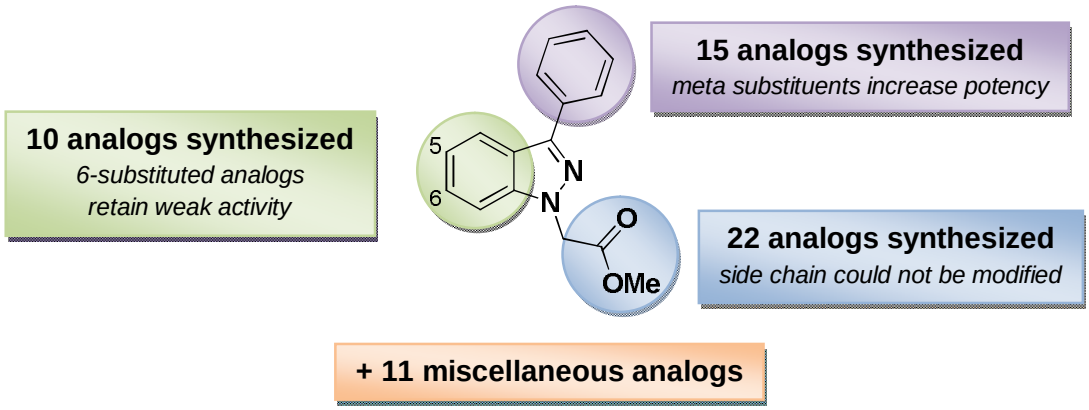
3.3 Scaffold/Moiety Chemical Liabilities

A search of PubChem for the probe (ML212) indicated that the probe has not been previously evaluated in any other assay. A structure-based search in SciFinder and Reaxys did not identify any publications or patents in which the probe appeared. The only potential chemical liability associated with the probe is possible hydrolysis of the methyl ester.

3.4 SAR Tables

Figure 8. Summary of analogs prepared to investigate the SAR profile of the HTS hit. Key SAR findings for each site of diversification are provided in italics.



In order to investigate the activity of this structural class, a collection of 58 structurally related analogs were synthesized and evaluated for their ability to reverse fluconazole resistance in the *C.albicans* test strains. **Figure 8** depicts the diversification points selected for modification. Three diversity points (highlighted in purple, blue, and green) were explored, and the number of analogs screened for each site is specified. Several analogs did not fit into these three clusters and are classified as "miscellaneous" analogs.

The biological assay data and physical properties of these analogs are presented in **Tables 1-6**. Characterization data (^{1}H NMR spectra and UPLC chromatograms) of these analogs are provided in **Appendix C.**

The initial hit from the HTS campaign described in Section 3.1 was methyl 3-phenyl-1*H*-indazole acetate (entry 1, **Table 1**). The side chain methyl ester of the hit was predicted to be susceptible to hydrolysis, and a series of ester and amide analogs were prepared accordingly to evaluate the necessity of this latent acid (**Table 1**). From this screen, it was determined that

replacement of the methyl ester with metabolically more stable derivatives was detrimental to cellular activity. The inactivity of these compounds suggested that perhaps the free acid was the active species. Unfortunately, when this predicted metabolite was prepared and tested, it also proved ineffective at countering fluconazole resistance in *C.albicans* (entry 12, **Table 1**).

Following the investigation of alternative esters and amides, efforts were undertaken to substantially modify the entire side chain. To this end, 10 analogs were prepared and tested (**Table 2**). The absence of the methyl acetate was not tolerated (entry 1, **Table 2**), and excision of any oxygen atoms from the ester system (entries 2-4, **Table 2**) was also not tolerated. Extension of the side chain length was similarly ineffective (entries 5 and 6, **Table 2**). Several compounds incorporating substitutions adjacent to the ester were also prepared (entries 7-9, **Table 2**). However, only the mono-methyl derivative (entry 7, **Table 2**) retained any activity in the cellular assay; but, this analog was less potent than the original hit. While the γ-lactone (entry 9, **Table 2**) was clean by NMR spectroscopy, UPLC indicated hydrolysis to the seco-acid readily occurs; this phenomenon may have contributed to the inactivity of this compound.

Finally, an oxazole conjugate was investigated as a possible ester surrogate. Although this derivative displayed very weak activity against CaCi-8, there was no measurable efficacy towards the less resistant CaCi-2 (entry 10, **Table 2**). Based on the results summarized in **Table 1** and **Table 2,** it appears that the methyl acetate system is critical to activity and further perturbation of this functionality is discouraged.

Table 3 presents several derivatives of the initial hit wherein the indazole core was modified to incorporate additional functional groups. This series of analogs focused predominantly on the 5- and 6-positions of the aromatic system. With regards to the 5-position (*i.e.* R_1), only the methyl or fluoro derivatives displayed low micromolar potency against CaCi-2 (entries 1 and 3, **Table 3**). Conversely, no activity was recorded for the methoxy or chloro counterparts (entries 2 and 4, **Table 3**). R_2, or the 6-position, appeared more amenable to manipulation as four of five conjugates demonstrated mild inhibitory effects towards CaCi-2 (entries 5-9, **Table 3**); only the 6-trifluoromethyl variant was inactive (entry 9, **Table 3**). It is notable that all of the 5- and 6-substituted variants were slightly active against the more resistant CaCi-8 strain (IC_{50} values 5.3 µM to 32.1 µM). SAR of the indazole core suggests the 6-position is an attractive site for future exploration, in particular for further optimization of aqueous solubility.

The last point of diversity explored was the 3-phenyl ring of the parent indazole scaffold. Fifteen analogs were synthesized to probe the SAR of this region, and the results are summarized in

Table 4 and **Table 5.** **Table 4** presents several nonaromatic analogs that were prepared to replace the benzene ring. Removal of the phenyl ring adversely affected potency; both the unsubstituted 1*H*-indazole and 3-methyl compounds were inactive in the primary assay (entries 1 and 3, **Table 4**). The 3-iodo derivative was likewise an ineffective chemosensitizing agent (entry 2, **Table 4**). Incidentally, this series of analogs boasted high PBS solubility, but sacrificed all cellular potency.

To complement the previous nonaromatic series, a collection of monosubstituted phenyl conjugates was prepared (**Table 5**). Substitution at the *para*-position was generally not productive (entries 1-6, **Table 5**); only the methoxy and fluoro compounds displayed measurable activity in the primary screen (entries 2 and 3, **Table 5**). This trend stands in stark contrast to modification of the *meta* site. All three indazoles bearing a *meta*-substituted phenyl ring were very potent chemosensitizers (entries 7-9, **Table 5**). Both the 3-(3-tolyl) and 3-(3-anisolyl)-1*H*- indazoles demonstrated nanomolar potency against CaCi-2 (IC_{50}= 650 nM and 440 nM, respectively) and low micromolar activity against CaCi-8 (IC_{50} = 1.3 µM and 1.2 µM, respectively). The 3-fluoro counterpart was marginally less effective (IC_{50} = 1.4 µM against CaCi-2 and 2.1 µM against CaCi-8). The *meta*-position was clearly the pivotal site for potency; the two *ortho*-substituted analogs were considerably less potent than the *meta*-series although there were small gains in solubility (entries 10-11, **Table 5**).

SAR evaluation of the original scaffold was concluded with a series of compounds encompassing larger structural perturbations (**Table 6**). For several analogs, the aromatic and acetate side chains were relocated to alternative sites around the indazole system (entries 1-6, **Table 6**). Not surprisingly, these drastic alterations failed to yield any beneficial increase to cellular potency. Entries 7 and 8 (**Table 6**) re-affirmed SAR trends previously observed – neither the 5- chloro indazole nor acetamide analogs were efficacious chemosensitizers (*cf.* **Table 3** and **Table 1,** respectively), and their corresponding hybrids were just as impotent. Various fragmented indazole systems did not exhibit any bioactivity when evaluated (entries 9-11, **Table 6**).

It can be concluded from these SAR studies that the methyl acetate side chain is invaluable to cellular bioactivity. Similarly, replacement of the 3-phenyl ring system with nonaromatic functionalities strongly attenuated potency. However, it was identified that *meta*-substituted phenyl rings were equipotent to or more potent than the original HTS hit. Modification of the *para*- or *ortho*-positions was generally counterproductive. The indazole core itself could tolerate functionalization at the 5-position to a limited degree while the adjacent 6-position could

accommodate substituents with only a small loss in activity. Most of the compounds evaluated exhibited poor solubility in PBS (<1 µM); the few compounds that were soluble were often completely inactive.

As a result of these synthetic efforts, it was possible to increase the potency of the original hit (CID 3243873) from 1.8 µM to 0.44 µM and led to the identification of 3-(3-methoxyphenyl)-1*H*-indazole as a new probe compound (ML212).

Table 1. Evaluation of 12 Synthetic Ester and Amide Analogs

SAR Analysis				Structure		Target Potency (μM) Mean ± S.E.M						Anti-target Potency(μM) Mean ± S.E.M		
Entry No.	CID	SID	Broad ID	*	R_1	CaCi-2[a]		CaCi-2[b]		CaCi-8[a]		Fibroblasts[b]		
						n^c	IC_{50}	n^c	IC_{50}	n^c	IC_{50}	n^c	IC_{50}	**
1	3243873	99245544	BRD-K37150847	S		8	1.86 ± 0.99	6	IA	8	2.53 ± 1.13	3	IA	ND
	PBS Solubility: 2.5 μM			*Plasma Protein Binding: 96%*		*Plasma Stability: <1.0%*				*Purity (UPLC): 97%*				
2	13330959	103910831	BRD-K17670538	S		3	IA	2	IA	3	IA	1	IA	ND
	PBS Solubility: <1.0 μM			*Plasma Protein Binding: 99%*		*Plasma Stability: 20%*				*Purity (UPLC): 99%*				
3	49835820	103910829	BRD-K51391281	S		3	IA	2	IA	3	IA	1	IA	ND
	PBS Solubility: <1.0 μM			*Plasma Protein Binding: 99%*		*Plasma Stability: 53%*				*Purity (UPLC): >99%*				
4	49835839	103910847	BRD-K85640613	S		3	IA	2	IA	3	IA	1	IA	ND
	PBS Solubility: <1.0 μM			*Plasma Protein Binding: >99%*		*Plasma Stability: 85%*				*Purity (UPLC): 100%*				
5	49835870	103910819	BRD-K39846706	S		1	IA	2	IA	1	IA	1	0.19	ND
	PBS Solubility: <1.0 μM			*Plasma Protein Binding: >99%*		*Plasma Stability: 2.0%*				*Purity (UPLC): 97%*				
6	49835857	103910827	BRD-K98546361	S		3	IA	2	IA	3	IA	1	IA	ND
	PBS Solubility: <1.0 μM			*Plasma Protein Binding: ND*		*Plasma Stability: 13%*				*Purity (UPLC): 74%*				
7	13330955	99245536	BRD-K79487335	S		3	IA	4	IA	3	IA	3	IA	ND
	PBS Solubility: 18.5 μM			*Plasma Protein Binding: 93%*		*Plasma Stability: >99%*				*Purity (UPLC): >99%*				

IA = Inactive; ND = Not determined
S = synthesized; P = purchased; **CaCi-2 to anti-target fold selectivity
[a] In the presence of fluconazole
[b] In the absence of fluconazole
[c] Number of replicates

Table 1. Evaluation of 12 Synthetic Ester and Amide Analogs, continued

SAR Analysis				Structure		Target Potency (μM) Mean ± S.E.M						Anti-target Potency(μM) Mean ± S.E.M		
Entry No	CID	SID	Broad ID	*	R_1	CaCi-2[a]		CaCi-2[b]		CaCi-8[a]		Fibroblasts[b]		
						n^c	IC_{50}	n^c	IC_{50}	n^c	IC_{50}	n^c	IC_{50}	**
8	49835882	103910836	BRD-K11584268	S		3	IA	2	IA	3	IA	1	IA	ND
	PBS Solubility: 325.5 μM			*Plasma Protein Binding: 98%*		*Plasma Stability: 98%*				*Purity (UPLC): >99%*				
9	20877577	99245533	BRD-K24457958	P		3	IA	4	IA	3	IA	3	IA	ND
	PBS Solubility: 5.4 μM			*Plasma Protein Binding: 97%*		*Plasma Stability: >99%*				*Purity (UPLC): 96%*				
10	20877568	99245564	BRD-K38464168	P		3	IA	4	IA	3	IA	3	IA	ND
	PBS Solubility: <1.0 μM			*Plasma Protein Binding: 95%*		*Plasma Stability: >99%*				*Purity (UPLC): 96%*				
11	20877557	99245566	BRD-K19712641	S		3	IA	4	IA	3	IA	3	IA	ND
	PBS Solubility: <1.0 μM			*Plasma Protein Binding: >99%*		*Plasma Stability: 97%*				*Purity (UPLC): >99%*				
12	5309553	99376506	BRD-K85225666	S		3	IA	3	IA	3	IA	2	IA	ND
	PBS Solubility: 534.6 μM			*Plasma Protein Binding: 97%*		*Plasma Stability: >99%*				*Purity (UPLC): 94%*				

IA = Inactive; ND = Not determined
S = synthesized; P = purchased; **CaCi-2 to anti-target fold selectivity
[a] In the presence of fluconazole
[b] In the absence of fluconazole
[c] Number of replicates

Table 2. Synthetic Replacements of the Methyl Acetate Side Chain (10 analogs)

SAR Analysis				Structure		Target Potency (μM) Mean ± S.E.M						Anti-target Potency (μM) Mean ± S.E.M		
Entry No.	CID	SID	Broad ID	*	R_1	CaCi-2[a]		CaCi-2[b]		CaCi-8[a]		Fibroblasts[b]		
						n[c]	IC_{50}	n[c]	IC_{50}	n[c]	IC_{50}	n[c]	IC_{50}	**
1	300385	99245532	BRD-K54502992	S		3	IA	4	IA	2	IA	2	IA	ND
	PBS Solubility: 91.0 μM			*Plasma Protein Binding: 98%*		*Plasma Stability: >99%*				*Purity (UPLC): 98%*				
2	49835816	103910823	BRD-K23177474	S		1	IA	2	IA	1	IA	1	IA	ND
	PBS Solubility: <1.0 μM			*Plasma Protein Binding: >99%*		*Plasma Stability: 98%*				*Purity (UPLC): 98%*				
3	49835825	103910830	BRD-K10798115	S		3	IA	2	IA	3	IA	1	IA	ND
	PBS Solubility: <1.0 μM			*Plasma Protein Binding: >99%*		*Plasma Stability: 70%*				*Purity (UPLC): 99%*				
4	49835848	103910810	BRD-K16789019	S		1	IA	2	IA	1	IA	1	IA	ND
	PBS Solubility: <1.0 μM			*Plasma Protein Binding: >99%*		*Plasma Stability: 81%*				*Purity (UPLC): >99%*				
5	49835835	103910835	BRD-K48860244	S		3	IA	2	IA	3	IA	1	IA	ND
	PBS Solubility: <1.0 μM			*Plasma Protein Binding: >99%*		*Plasma Stability: 74%*				*Purity (UPLC): >99%*				
6	49835881	103910822	BRD-K48854225	S		1	IA	2	IA	1	IA	1	IA	ND
	PBS Solubility: <1.0 μM			*Plasma Protein Binding: >99%*		*Plasma Stability: 57%*				*Purity (UPLC): >99%*				

Table 2. Synthetic Replacements of the Methyl Acetate Side Chain (10 analogs), continued

SAR Analysis				Structure		Target Potency (μM) Mean ± S.E.M						Anti-target Potency (μM) Mean ± S.E.M		
Entry No.	CID	SID	Broad ID	*	R_1	CaCi-2[a]		CaCi-2[b]		CaCi-8[a]		Fibroblasts[b]		
						n[c]	IC_{50}	n[c]	IC_{50}	n[c]	IC_{50}	n[c]	IC_{50}	**
7	49835813	103910834	BRD-M84860065	S		2	6.56 ± 0.79	2	IA	2	15.9 ± 2.2	1	IA	ND
	PBS Solubility: <1.0 μM			*Plasma Protein Binding: >99%*		*Plasma Stability: 61%*				*Purity (UPLC): 99%*				
8	49835873	103910811	BRD-K04729536	S		1	IA	2	IA	1	IA	1	IA	ND
	PBS Solubility: <1.0 μM			*Plasma Protein Binding: >99%*		*Plasma Stability: 63%*				*Purity (UPLC): >99%*				
9	49835834	103910812	BRD-A41230630	S		1	IA	2	IA	1	IA	1	IA	ND
	PBS Solubility: <1.0 μM			*Plasma Protein Binding: ND*		*Plasma Stability: <1.0 %*				*Purity (UPLC): 65%*				
10	49835836	103910824	BRD-K71142328	S		3	IA	2	IA	3	34.2 ± 7.9	1	IA	ND
	PBS Solubility: <1.0 μM			*Plasma Protein Binding: >99%*		*Plasma Stability: 97%*				*Purity (UPLC): >99%*				

IA = Inactive; ND = Not determined; S = synthesized; P = purchased; **CaCi-2 to anti-target fold selectivity
[a] In the presence of fluconazole
[b] In the absence of fluconazole
[c] Number of replicates

Table 3. Biological and Physical Properties of Substituted Indazoles (10 analogs)

SAR Analysis				Structure			Target Potency (μM) Mean ± S.E.M						Anti-target Potency(μM) Mean ± S.E.M		
Entry No.	CID	SID	Broad ID	*	R_1	R_2	CaCi-2[a]		CaCi-2[b]		CaCi-8[a]		Fibroblasts[b]		
							n^c	IC_{50}	n^c	IC_{50}	n^c	IC_{50}	n^c	IC_{50}	**
1	49835874	103910845	BRD-K76441112	S	Me	H	3	3.28 ± 1.06	2	IA	3	5.33 ± 0.72	1	69.5	21
	PBS Solubility: <1.0 μM			*Plasma Protein Binding: 95%*			*Plasma Stability: <1.0%*					*Purity (UPLC): 97%*			
2	49835837	103910851	BRD-K03923773	S	OMe	H	3	IA	2	IA	3	18.5 ± 7.6	1	IA	ND
	PBS Solubility: <1.0 μM			*Plasma Protein Binding: <1.0%*			*Plasma Stability: <1.0%*					*Purity (UPLC): >99%*			
3	49835889	103910846	BRD-K89892330	S	F	H	3	4.63 ± 0.30	2	IA	3	6.98 ± 0.69	1	IA	ND
	PBS Solubility: <1.0 μM			*Plasma Protein Binding: 96%*			*Plasma Stability: 1.0%*					*Purity (UPLC): 82%*			
4	9551137	99245555	BRD-K17052831	S	Cl	H	3	IA	4	IA	3	9.84 ± 1.03	3	IA	ND
	PBS Solubility: <1.0 μM			*Plasma Protein Binding: >99%*			*Plasma Stability: <1.0%*					*Purity (UPLC): 93%*			
5	49835872	103910833	BRD-K17372907	S	H	Me	3	3.14 ± 0.99	2	IA	2	9.62 ± 0.34	1	31.6	10
	PBS Solubility: <1.0 μM			*Plasma Protein Binding: 98%*			*Plasma Stability: 6.5%*					*Purity (UPLC): 87%*			
6	49835812	103910841	BRD-K59966621	S	H	OMe	3	7.37 ± 1.49	2	IA	3	12.7 ± 0.6	1	0.80	0.01
	PBS Solubility: <1.0 μM			*Plasma Protein Binding: 38%*			*Plasma Stability: 1.3%*					*Purity (UPLC): 93%*			
7	49835815	103910832	BRD-K21644500	S	H	F	3	5.10 ± 0.96	2	IA	3	8.34 ± 4.30	1	IA	ND
	PBS Solubility: <1.0 μM			*Plasma Protein Binding: 97%*			*Plasma Stability: 1.1%*					*Purity (UPLC): 92%*			

Table 3. Biological and Physical Properties of Substituted Indazoles (10 analogs), continued

SAR Analysis				Structure			Target Potency (μM) Mean ± S.E.M						Anti-target Potency(μM) Mean ± S.E.M		
Entry No.	CID	SID	Broad ID	*	R_1	R_2	CaCi-2[a]		CaCi-2[b]		CaCi-8[a]		Fibroblasts[b]		
							n[c]	IC_{50}	n[c]	IC_{50}	n[c]	IC_{50}	n[c]	C_{50}	**
8	49835818	103910839	BRD-K77651440	S	H	Cl	3	3.79 ± 1.97	2	IA	3	8.57 ± 0.69	1	29.9	7.9
	PBS Solubility: <1.0 μM			*Plasma Protein Binding: 18%*			*Plasma Stability: 90%*					*Purity (UPLC): 92%*			
9	49835849	103910808	BRD-K95485521	S	H	CF_3	4	IA	3	IA	4	32.1 ± 17.4	1	IA	ND
	PBS Solubility: <1.0 μM			*Plasma Protein Binding: 62%*			*Plasma Stability: 1.7%*					*Purity (UPLC): 95%*			
10	49835842	103910809	BRD-K32112425	S			1	IA	2	IA	1	IA	1	IA	ND
	PBS Solubility: <1.0 μM			*Plasma Protein Binding: >99%*			*Plasma Stability: 50%*					*Purity (UPLC): >99%*			

IA = Inactive; ND = Not determined;S = synthesized; P = purchased;
**CaCi-2 to anti-target fold selectivity
[a] In the presence of fluconazole
[b] In the absence of fluconazole
[c] Number of replicates

IA = Inactive; ND = Not determined
S = synthesized; P = purchased; **CaCi-2 to anti-target fold selectivity
[a] In the presence of fluconazole
[b] In the absence of fluconazole
[c] Number of replicates

Table 4. Alkyl Replacements of the 3-phenyl Ring (3 analogs)

SAR Analysis				Structure		Target Potency (μM) Mean ± S.E.M						Anti-target Potency(μM) Mean ± S.E.M		
Entry No.	CID	SID	Broad ID	*	R_1	CaCi-2[a]		CaCi-2[b]		CaCi-8[a]		Fibroblasts[b]		
						n[c]	IC_{50}	n[c]	IC_{50}	n[c]	IC_{50}	n[c]	IC_{50}	**
1	49835852	103910820	BRD-K65977579	S		1	IA	2	IA	1	IA	1	0.74	ND
	PBS Solubility: 154.7 μM			*Plasma Protein Binding: <1.0%*		*Plasma Stability: <1.0%*				*Purity (UPLC): >99%*				
2	49835832	103910818	BRD-K67191613	S		1	IA	2	IA	1	18.8	1	0.53	ND
	PBS Solubility: 143.5 μM			*Plasma Protein Binding: 98%*		*Plasma Stability: 1.3%*				*Purity (UPLC): 91%*				
3	46856254	99245571	BRD-K61868295	S		3	IA	4	IA	3	21.3 ± 8.1	3	IA	ND
	PBS Solubility: 287.5 μM			*Plasma Protein Binding: 42%*		*Plasma Stability: 3.0%*				*Purity (UPLC): >99%*				

IA = Inactive; ND = Not determined
S = synthesized; P = purchased; **CaCi-2 to anti-target fold selectivity
[a] In the presence of fluconazole
[b] In the absence of fluconazole
[c] Number of replicates

Table 5. Effect of Substitution on the 3-phenyl Ring System (12 analogs)

SAR Analysis				Structure		Target Potency (μM) Mean ± S.E.M						Anti-target Potency (μM) Mean ± S.E.M		
Entry No.	CID	SID	Broad ID	*	R_1	CaCi-2[a]		CaCi-2[b]		CaCi-8[a]		Fibroblasts[b]		
						n^c	IC_{50}	n^c	IC_{50}	n^c	IC_{50}	n^c	IC_{50}	**
1	46856255	99245540	BRD-K66205757	S	4-Me	3	IA	4	IA	3	11.0 ± 3.2	3	IA	ND
	PBS Solubility: 1.7 μM				*Plasma Protein Binding: 96%*			*Plasma Stability: 4.1%*				*Purity (UPLC): 95%*		
2	49835864	103910840	BRD-K00916099	S	4-OMe	3	4.23 ± 1.67	1	IA	3	8.30 ± 0.20	1	1.02	0.24
	PBS Solubility: <1.0 μM				*Plasma Protein Binding: 94%*			*Plasma Stability: 2.7%*				*Purity (UPLC): 97%*		
3	49835876	103910817	BRD-K83306461	S	4-F	4	6.26 ± 1.63	3	IA	4	10.2 ± 4.0	1	IA	ND
	PBS Solubility: <1.0 μM				*Plasma Protein Binding: 93%*			*Plasma Stability: <1.0%*				*Purity (UPLC): 95%*		
4	46856253	99245538	BRD-K13053491	S	4-Cl	4	IA	4	IA	3	11.6 ± 4.7	3	IA	ND
	PBS Solubility: <1.0 μM				*Plasma Protein Binding: >99%*			*Plasma Stability: 6.4%*				*Purity (UPLC): 95%*		
5	49835854	103910826	BRD-K89702943	S	4-CF_3	3	IA	3	IA	1	IA	1	0.68	ND
	PBS Solubility: <1.0 μM				*Plasma Protein Binding: 97%*			*Plasma Stability: 17%*				*Purity (UPLC): 97%*		
6	49835863	103910815	BRD-K09194947	S	4-CN	1	IA	2	IA	1	20.0	1	IA	ND
	PBS Solubility: <1.0 μM				*Plasma Protein Binding: 92%*			*Plasma Stability: <1.0%*				*Purity (UPLC): 92%*		
7	49835823	103910848	BRD-K33918068	S	3-Me	3	0.65 ± 0.25	2	IA	3	1.34 ± 0.26	1	IA	ND
	PBS Solubility: <1.0 μM				*Plasma Protein Binding: 98%*			*Plasma Stability: 4.7%*				*Purity (UPLC): 97%*		
8	49835877	103910843	BRD-K14324645	S	3-OMe	3	0.44 ± 0.08	2	IA	3	1.21 ± 0.17	1	IA	ND
	PBS Solubility: 3.6 μM				*Plasma Protein Binding: 95%*			*Plasma Stability: 2.6%*				*Purity (UPLC): 93%*		

Table 5. Effect of Substitution on the 3-phenyl Ring System (12 analogs), continued

SAR Analysis				Structure		Target Potency (μM) Mean ± S.E.M						Anti-target Potency (μM) Mean ± S.E.M		
Entry No.	CID	SID	Broad ID	*	R_1	CaCi-2[a]		CaCi-2[b]		CaCi-8[a]		Fibroblasts[b]		
						n[c]	IC_{50}	n[c]	IC_{50}	n[c]	IC_{50}	n[c]	IC_{50}	**
9	49835858	103910816	BRD-K34975656	S	3-F	3	1.41 ± 0.08	3	IA	3	2.14 ± 0.44	1	12.9	9.1
	PBS Solubility: <1.0 μM			*Plasma Protein Binding: 96%*		*Plasma Stability: <1.0%*					*Purity (UPLC): 95%*			
10	49835865	103910813	BRD-K13238786	S	*2-Me*	3	5.50 ± 0.65	3	IA	3	7.25 ± 3.52	1	IA	ND
	PBS Solubility: 3.7 μM			*Plasma Protein Binding: <1.0%*		*Plasma Stability: <1.0%*					*Purity (UPLC): 93%*			
11	49835850	103910838	BRD-K39093020	S	2-OMe	3	6.21 ± 1.73	2	IA	3	10.2 ± 1.8	1	1.04	0.17
	PBS Solubility: 26.7 μM			*Plasma Protein Binding: 92%*		*Plasma Stability: <1.0%*					*Purity (UPLC): 97%*			
12	49835868	103910849	BRD-K58348307	S		3	IA	2	IA	1	IA	1	IA	ND
	PBS Solubility: 154.6 μM			*Plasma Protein Binding: ND*		*Plasma Stability: <1.0%*					*Purity (UPLC): 93%*			

IA = Inactive; ND = Not determined
S = synthesized; P = purchased; **CaCi-2 to anti-target fold selectivity
[a] In the presence of fluconazole
[b] In the absence of fluconazole
[c] Number of replicates

Table 6. Miscellaneous Analogs of the 3-phenyl-1H-indazole Scaffold (11 analogs)

SAR Analysis						Target Potency (μM) Mean ± S.E.M						Anti-target Potency (μM) Mean ± S.E.M		
Entry No.	CID	SID	Broad ID	*	Structure	CaCi-2[a]		CaCi-2[b]		CaCi-8[a]		Fibroblasts[b]		
						n[c]	IC_{50}	n[c]	IC_{50}	n[c]	IC_{50}	n[c]	IC_{50}	**
1	12312246	99245565	BRD-K08771473	S		3	IA	4	IA	3	IA	3	IA	ND
	PBS Solubility: <1.0 μM			*Plasma Protein Binding: >99%*				*Plasma Stability: 93%*				*Purity (UPLC): >99%*		
2	100493	99245554	BRD-K67484673	S		3	IA	4	IA	2	IA	3	IA	ND
	PBS Solubility: 516.1 μM			*Plasma Protein Binding: >99%*				*Plasma Stability: >99%*				*Purity (UPLC): 99%*		
3	46856252	99245530	BRD-K96609729	S		3	IA	4	IA	3	IA	3	IA	ND
	PBS Solubility: <1.0 μM			*Plasma Protein Binding: >99%*				*Plasma Stability: 70%*				*Purity (UPLC): 99%*		
4	14420692	99245552	BRD-K03169838	S		3	IA	3	IA	3	IA	3	IA	ND
	PBS Solubility: 502.6 μM			*Plasma Protein Binding: >99%*				*Plasma Stability: 96%*				*Purity (UPLC): 95%*		

Table 6. Miscellaneous Analogs of the 3-phenyl-1H-indazole Scaffold (11 analogs), continued

SAR Analysis						Target Potency (μM) Mean ± S.E.M						Anti-target Potency (μM) Mean ± S.E.M		
Entry No	CID	SID	Broad ID	*	Structure	CaCi-2[a]		CaCi-2[b]		CaCi-8[a]		Fibroblasts[b]		
						n[c]	IC_{50}	n[c]	IC_{50}	n[c]	IC_{50}	n[c]	IC_{50}	**
5	46378661	99245545	BRD-K77711392	P		3	IA	4	IA	3	IA	3	IA	ND
	PBS Solubility: 529.1 μM			*Plasma Protein Binding: 52%*				*Plasma Stability: >99%*				*Purity (UPLC): 96%*		
6	4131200	103023261	BRD-K61757267	S		3	IA	3	IA	3	IA	2	IA	ND
	PBS Solubility: 438.2 μM			*Plasma Protein Binding: 95%*				*Plasma Stability: 46%*				*Purity (UPLC): 98%*		
7	20877381	99245558	BRD-K89784498	P		3	IA	4	IA	3	IA	3	IA	ND
	PBS Solubility: 8.0 μM			*Plasma Protein Binding: 96%*				*Plasma Stability: 99%*				*Purity (UPLC): >99%*		

Table 6. Miscellaneous Analogs of the 3-phenyl-1H-indazole Scaffold (11 analogs), continued

SAR Analysis						Target Potency (μM) Mean ± S.E.M						Anti-target Potency (μM) Mean ± S.E.M		
Entry No	CID	SID	Broad ID	*	Structure	CaCi-2[a]		CaCi-2[b]		CaCi-8[a]		Fibroblasts[b]		
						n[c]	IC_{50}	n[c]	IC_{50}	n[c]	IC_{50}	n[c]	IC_{50}	**
8	20877322	99245547	BRD-K50197321	P		3	IA	4	IA	2	IA	3	IA	ND
	PBS Solubility: <1.0 μM			*Plasma Protein Binding: >99%*				*Plasma Stability: 97%*				*Purity (UPLC): >99%*		
9	46856251	99245549	BRD-K42988725	S		3	IA	4	IA	3	IA	3	IA	ND
	PBS Solubility: 494.5 μM			*Plasma Protein Binding: 63%*				*Plasma Stability: >99%*				*Purity (HPLC): 98%*		
10	9855970	103910821	BRD-K20739871			1	IA	2	IA	1	IA	1	IA	ND
	PBS Solubility: <1.0 μM			*Plasma Protein Binding: 98%*				*Plasma Stability: 89%*				*Purity (UPLC): 98%*		
11	49835869	103910814	BRD-K52595598			1	IA	2	IA	1	IA	1	IA	ND
	PBS Solubility: <1.0 μM			*Plasma Protein Binding: >99%*				*Plasma Stability: 79%*				*Purity (UPLC): >99%*		

IA = Inactive; ND = Not determined;S = synthesized; P = purchased; **CaCi-2 to anti-target fold selectivity
[a] In the presence of fluconazole; [b] In the absence of fluconazole; [c] Number of replicates

3.5 Cellular Activity

All assays were performed with whole cells. A murine 3T3 fibroblast mammalian cell toxicity assay was included as a secondary screen. Experimental details are provided above in Section 2.1.2. The probe (ML212) clearly met the established probe criteria specified for this project (**Table 7**).

Table 7. Comparison of Probe to Project Criteria

No.	Property	Requirement	Probe
1	CaCi-2 IC_{50} with 8 µg/ml fluconazole	Less than 1 µM	0.44 µM
2	CaCi-8 IC_{50} with 8 µg/ml fluconazole	Less than 50 µM	1.21 µM
3	CaCi-2 IC_{50} without fluconazole	Greater than 10 µM	>26 µM
4	Mammalian fibroblast IC_{50}	At least 10-fold greater than (1)	>59-fold

3.6 Profiling Assays

The probe (ML212) was evaluated for inhibitory activity against calcinuerin and Hsp90. Both proteins have been linked to the acquisition and maintenance of antifungal drug resistance in *C. albicans*. Assay results indicated the probe was a mild inhibitor of the Hsp90 pathway (IC_{50} = 4.18 µM). Additional details are provided below in Section 4.2.

4 Discussion

4.1 Comparison to existing art and how the new probe is an improvement

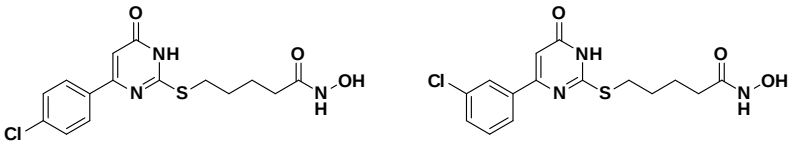
Investigation into relevant prior art entailed searching the following databases: SciFinder, Reaxys, PubChem, PubMed, US Patent and Trademark Office (USPTO), PatFT, AppFT, and World Intellectual Property Organization (WIPO). The search terms applied and hit statistics are provided in **Table 8**. The searches were performed on and are current as of February 7, 2011.

Table 8. Search Strings and Databases Employed in the Prior Art Search

Search String	Database	Hits Found
“fluconazole resistance”	SciFinder	3071
“fluconazole sensitivity”	SciFinder	702
“fluconazole chemosensitizer”	SciFinder	12
“fluconazole resistance”	Reaxys	162
“fluconazole AND sensitivity”	Reaxys	20
“fluconazole AND chemosensitize”	Reaxys	1
“fluconazole resistance”	PubChem Bioassay	250
“fluconazole sensitivity”	PubChem Bioassay	34
“fluconazole chemosensitize”	PubChem Bioassay	0
“fluconazole resistance”	PubMed	1923
“fluconazole sensitivity”	PubMed	2153
“fluconazole chemosensitize”	PubMed	1
“fluconazole resistance”	USPTO PatFT	16
“fluconazole resistance”	USPTO AppFT	28
“fluconazole sensitivity”	WIPO	1225

Several compounds have been previously identified as chemosensitizers, increasing the susceptibility of various *C.albicans* strains to fluconazole treatment (2-7). Of these, the most potent belonged to a series of HDAC inhibitors reported by Mai et al. (7). Depicted in **Figure 9**, these compounds are uracil-derived hydroxamic acids and exhibited MIC values ranging from 1.2 µM to 2.8 µM when combined with fluconazole. When tested in the absence of fluconazole, neither compound demonstrated activity against *C.albicans* at concentrations up to 368 µM. However, the potent activity of these compounds against murine HDAC1 (IC_{50} <51 nM), combined with the antiproliferative activity of similar analogs in human cells (8,9), indicates they would not possess any significant species selectivity. The absence of selectivity disqualified compounds **4** and **5** as probe candidates. In addition, neither compound could be readily obtained for direct comparison to ML212.

Figure 9. Chemosensitizers for Reversing Fluconazole Resistance (MIC=1.2-2.8 µM)



The probe (ML212) has a potency of 440 nM against the *C.albicans* strain CaCi-2, and does not exhibit any effect in the absence of fluconazole or against mammalian fibroblasts.

4.2 Mechanism of Action Studies

Previous studies have demonstrated that functional Hsp90 and calcineurin are critical to drug resistance. In order to determine if ML212 affects the Hsp90 pathway or calcineurin itself, secondary assays were established.

Potential inhibition of the Hsp90-based chaperone machinery was evaluated using yeast reporter assays involving the glucocorticoid hormone receptor and the tyrosine kinase, v-Src. Both of these well established client proteins depend heavily on Hsp90 for their function. Refer to Section 2.1.4 for method details.

Potential inhibition of calcineurin function was evaluated in yeast carrying a construct encoding calcineurin-dependent response elements (CDRE) driving expression of a reporter enzyme. Reporter activity with or without the prior addition of test compounds was measured following challenge with the stressor $CaCl_2$.

The probe (ML212) exhibited Hsp90 pathway inhibition with IC_{50} = 4.18 µM in the *S. cerevisiae* model. The inactivity of the compound in the mammalian fibroblast assay (IC_{50} > 26 µM) suggests that ML212 may be a *fungal-selective* inhibitor of the Hsp90 pathway. Further investigations will evaluate this possibility and determine if this is the mode of action in *C.albicans*.

4.3 Planned Future Studies

The goal of probe optimization is to generate potent, fungal-specific agents for future mechanistic studies and target identification projects. The Lindquist lab is currently performing *C.albicans* resistance studies with ML212, and preliminary results suggest that generation of a ML212-resistant strain is possible. Successful induction of resistance would enable further investigation of ML212's cellular target through the use of powerful genetic approaches that are

available, including genome-wide, over expression, and deletion libraries, both in parallel arrayed format and pooled bar-coded format. To complement genetic approaches, affinity precipitation and proteomic approaches based on Stable Isotope Labeling with Amino acids in Cell culture (SILAC) technology, which are already being employed in other projects, may be used. The drug metabolism/pharmacokinetic (DMPK) properties of the probe can be further optimized, and the probe (ML212) could be studied in well-established mouse models of invasive, drug-resistant *Candida* fungemia, which are available and have been employed in the Whitehead Institute in conjunction with the laboratory of Gerald Fink. There is currently an IACUC-approved protocol in place to enable such experimentation in future work and to evaluate the therapeutic potential of probes generated by this screen.

5 References

1. Cowen LE, Lindquist S. Hsp90 potentiates the rapid evolution of new traits: drug resistance in diverse fungi. Science 2005 Sep 30; 309(5744): 2185-89. PubMed PMID: 16195452

2. DiGirolamo JA, Li X.-C, Jacob MR, Clark AM, Ferreira D. Reversal of fluconazole resistance by sulfated sterols from the marine sponge Topsentia sp. J Nat Prod. 2009 Aug; 72(8):1524-28.PubMed PMID: 19653640

3. Cernicka J, Kozovska Z, Hnatova M, Valachovic M, Hapala I, Riedl Z, Hajós G, Subik J. Chemosensitisation of drug-resistant and drug-sensitive yeast cells to antifungals. Int J Antimicrobial Agents. 2007 Feb; 29(2):170-8. PubMed PMID: 17204400; Epub 2007 Jan 3; doi:10.1016/j.ijantimicag.2006.08.037

4. Gamarra S, Rocha EM, Zhang Y.-Q, Park S, Rao R, Perlin DS. Mechanism of the synergistic effect of amiodarone and fluconazole in Candida albicans. Antimicrob Agents Chemother 2010 May;54(5):1753-61. PubMed PMID: 20194694; PMCID: PMC2863688;Epub 2010 Mar 1.

5. Guo X.-L, Leng P, Yang Y, Yu L.-G, Lou H.-X. Plagiochin E, a botanic-derived phenolic compound, reverses fungal resistance to fluconazole relating to the efflux pump J Appl Microbio 2008 Mar;104(3):831-38. Epub 2008 Jan 9; doi: 10.1111/j.1365-2672.2007.03617.x

6. Courchesne WE. Characterization of a novel, broad-based fungicidal activity for the antiarrhythmic drug amiodarone. J Pharmacol Exp Ther 2002 Jan 1; 300:195-99; Epub doi:10.1124/jpet.300.1.195

7. Mai A, Rotili D, Massa S, Brosch G, Simonetti G, Passariello C, Palamara AT. Discovery of uracil-based histone deacetylase inhibitors able to reduce acquired antifungal resistance and trailing growth in Candida albicans. Bioorg Med Chem Lett 2007 Mar 1; 17(5):1221-25. PubMed PMID: 17196388; Epub 2006 Dec 12.

8. Mai A, Massa S, Rotili D, Pezzi R, Bottoni P, Scatena R, Meraner J, Brosch G. Exploring the connection unit in the HDAC inhibitor pharmacophore model: Novel uracil-based hydroxamates. Bioorg Med Chem Lett 2005 Nov 1; 15(21):4656-61. PubMed PMID: 16165353.

9. Mai A, Massa S, Rotili D, Simeoni S, Ragno R, Botta G, Nebbioso A, Miceli M, Altucci L, Brosch G. Synthesis and biological properties of novel, uracil-containing histone deacetylase inhibitors. J Med Chem. 2006 Oct 5;49(20):6046-56. PubMed PMID: 17004718

10. Redding S, Smith J, Farinacci G, Rinaldi M, Fothergill A, Rhine-Chalberg J, Pfaller M. Resistance of *Candida albicans* to fluconazole during treatment of oropharyngeal candidiasis in a patient with AIDS: documentation by in vitro susceptibility testing and DNA subtype analysis. Clin Infect Dis 1994 Feb 1; 18(2): 240-42. PubMed PMID: 8161633

11. Salovich JM, Lindsley CW, Hopkins CR. Synthesis of 2,3-diarylsubstituted indazoles utilizing a Suzuki cross-coupling/deprotection/N-arylation sequence. Tetrahedron Lett. 2010 Jul 21; 51(29):3796-3799. PubMed PMID: 20606711

12. Bouissane L, El Kazzouli S, Léonce S, Pfeiffer B, Rakib EM, Khouili M, Guillaumet G. Synthesis and biological evaluation of N-(7-indazolyl)benzenesulfonamide derivatives as potent cell cycle inhibitors. Bioorg Med Chem. 2006 Feb 15; 14(4):1078-88. PubMed PMID: 16274996

13. Baldwin IR, Bamborough P, Christopher JA, Kerns JK, Longstaff T, Mill DD. Indole derivatives and use thereof as kinase inhibitors in particular IKK2 inhibitors. Int. Pat. Appl. WO 2005/067923 A1, 2005.

Appendix A: Assay Summary Table

Table A1. Summary of Completed Assays and AIDs

PubChem AID	Type	Target	Concentration Range (μM)	Samples Tested
1979	Primary	CaCi-2 growth inhibition	9.5	302509
2467	Confirmatory	CaCi-2 growth inhibition	3.8 – 0.03	1654
488836	Confirmatory (powder)	CaCi-2 growth inhibition	26 – 0.1	30
493089	Analogs	CaCi-2 growth inhibition	12 – 0.05	54
493081	Analogs 2	CaCi-2 growth inhibition	12 – 0.05	29
493080	Analogs 3	CaCi-2 growth inhibition	12 – 0.05	128
493150	Analogs 4	CaCi-2 growth inhibition	12 – 0.05	33
2327	Counterscreen	Fibroblast toxicity	16 – 0.12	1654
488809	Counterscreen (powder)	Fibroblast toxicity	26 – 0.1	44
493099	Counterscreen (analogs)	Fibroblast toxicity	26 – 0.1	64
493147	Counterscreen (analogs 2)	Fibroblast toxicity	6 – 0.05	108
2423	Orthogonal	CaCi-8 growth inhibition	16 – 0.12	1654
488807	Orthogonal (powder)	CaCi-8 growth inhibition	26 – 0.1	44
493064	Orthogonal (analogs)	CaCi-8 growth inhibition	26 – 0.1	64
493069	Orthogonal (analogs 2)	CaCi-8 growth inhibition	26 – 0.1	27
493082	Orthogonal (analogs 3)	CaCi-8 growth inhibition	26 – 0.1	29
493149	Orthogonal (analogs 3)	CaCi-8 growth inhibition	26 – 0.1	33
2387	Secondary	CaCi-2 growth inhibition	16 – 0.12	350
488802	Secondary (powder)	CaCi-2 growth inhibition	26 – 0.1	30
493070	Secondary (analogs2)	CaCi-2 growth inhibition	26 – 0.1	27
493157	Secondary (analogs3)	CaCi-2 growth inhibition	26 – 0.1	29
493134	Secondary (analogs3)	CaCi-2 growth inhibition	26 – 0.1	33
2400	Secondary	Hsp90	16 – 0.12	350

PubChem AID	Type	Target	Concentration Range (μM)	Samples Tested
504390	Secondary (analogs)	Hsp90	26 – 0.1	18
2388	Secondary	Calcineurin	16 – 0.12	350
2007	Summary	NA	NA	NA

Appendix B: Detailed Assay Protocol

Primary CaCi-2 (AID No. 1979) and CaCi-2 Dose-Response Retest (AID Nos. 2467, 488836)

Materials and Reagents:

Clear, flat bottom, black, 384-well plates (Corning Catalog no. 3712BC Lot no. 35808016); Geldanamycin (AG Scientific, catalog no. G-1047) 15 mM stock solution in DMSO; Fluconazole (Sequoia Research Products Ltd) 2 mg/ml stock solution in PBS; Pen/Strep (Gibco Catalog no.10378-016; Lot no. 21040170) 100X in PBS; Alamar Blue (AG Scientific Catalog no.DAL 1100; Lot no.151016SA); PBS without Calcium and Magnesium (Cellgro Catalog no. 21-040-CV)

Synthetic Defined Growth Medium

RPMI 1640 medium, (powder without sodium bicarbonate; Invitrogen Catalog no. 31800-089; Lot no. 648072); Uridine 8 mg/ml in water (Sigma Catalog no. U3750, Lot no. 028KO760); Glucose 40% (w/v) in water (Sigma Catalog no. G-5400); MOPS Buffer (Sigma Catalog no. M-1254; Lot no. 098K0033)

1) Prepare 1X RPMI medium by dissolving 10.4 grams powdered medium in 800 ml water.
2) Add 34.52 g MOPS. While stirring, adjust pH to 7.0 with 10 N NaOH.
3) Add 10 ml uridine solution, 50 ml glucose solution, adjust final volume to 1000 ml. Filter sterilize.

Fungal Inoculum

Test Strain: *C. albicans* CaCi-2

1) Inoculate 500 µlL of strain from cryopreserved stock into 250 ml shaker flask containing 30 ml growth medium. Shake at 30 °C overnight.
2) Read OD 600 of 1 ml fungal culture in a cuvette using a standard optical density reader (Eppendorf BioPhotometer Plus), with growth medium as a background blank.
3) Dilute to desired volume of fungal inoculums according to the following formula: (1/OD measured) X (Desired Final Volume of Inoculum) X 0.3 = Volume of fungal culture (µl) to add to desired volume of growth medium. When added to media in wells, this yields a calculated starting OD of the fungal inoculum of 0.00015.

Procedures:

1) Add fluconazole stock solution to fungal inoculum to achieve a final concentration of 8 µg/ml.
2) Add Pen/Strep at 0.1 ml per 10 ml media (1% v/v).
3) Use a Thermo Combi nL to dispense 20 µl/well of assay media into all wells.
4) Pin 25 nL test compound from compound plates into assay plates using CyBi-Well pin tool.
5) Dispense 20 µl/well of culture into the assay media in all wells.
6) Incubate plates in a humidified (90% humidity) Liconic incubator at 37 °C without agitation for 48 hours.
7) Dilute Alamar Blue Reagent 1:40 in Ca/Mg-free PBS.
8) To all plates, add 5 µl/well of the diluted Alamar to a final dilution factor of 1:200.
9) Incubate the plates for an additional 2 hours.
10) Read the Relative Fluorescence Intensity (RFU) of wells on a standard plate reader as a measure of relative fungal growth. Envision (Perkin Elmer) plate reader set-up: Ex 544 nm, Em 590 nm, Bandwidth 12 nm, Top read.

Orthogonal Resistant Strain Dose Response (AID Nos. 2423, 488807, 493064, 493069, 493082, 493149)

Materials and Reagents:

Clear, flat bottom, black 384-well plates (Corning Catalog no. 3712BC; Lot no. 35808016); Geldanamycin (AG Scientific Catalog no. G-1047) 15 mM stock solution in DMSO; Pen/Strep (Gibco Catalog no.10378-016;Lot no.21040170) 100X in PBS; Fluconazole (Sigma Catalog no.F829-100MG;Lot no. 098K4715) 2 mg/ml stock solution in PBS; Alamar Blue (AG Scientific Catalog no. DAL1100; Lot no.151016SA);PBS w/o Calcium and Magnesium (Cellgro Catalog no. 21-040-CV)

Synthetic Defined Growth Medium

RPMI 1640 medium, (powder without sodium bicarbonate; Invitrogen Catalog no. 31800-089, Lot no.648072); Uridine 8 mg/ml in water (Sigma Catalog no. U3750; Lot no. 028K0760); Glucose 40% (w/v) in water (Sigma Catalog no. G-5400); MOPS Buffer (Sigma Catalog no. M-1254; Lot no. 098K0033)

1) Prepare 1X RPMI medium by dissolving 10.4 g powdered medium in 800 ml water.
2) Add 34.52 g MOPS. While stirring, adjust pH to 7.0 with 10N NaOH.
3) Add 10 ml uridine solution, 50 ml glucose solution, adjust final volume to 1000 ml. Filter sterilize

Fungal Inoculum

Test Strain: *C. albicans* CaCi8 (10)

1) Inoculate 500 µl of strain from cryopreserved stock into 250 ml shaker flask containing 30 ml growth medium. Shake at 30 °C overnight (16 hours).
2) Read OD 600 of 1 ml of fungal culture in a cuvette using a standard optical density reader (Eppendorf BioPhotometer Plus), with growth medium as a background blank.
3) Dilute to a desired volume of fungal inoculum according to following formula: (1/OD measured) X (Desired Final Volume of Inoculum) X 0.3 = Volume of fungal culture (µl) to add to desired volume of growth medium. When added to media in wells, this yields a calculated starting OD of the fungal inoculum of 0.00015.

Procedures:

1) Add fluconazole stock solution to fungal inoculum to achieve 8 µg/ml.
2) Add Pen/Strep to media to 1% concentration.
3) Use a Thermo Combi nL to dispense 20 µl/well of assay media into all wells.
4) Dispense geldanamycin in positive control wells using Thermo Combi nL for a final concentration of 3 µM.
5) Then, pin 100 nl of test compound from compound plates into assay plates using a CyBi-Well pin tool.
6) Dispense 20 µl/well of culture into the assay media in all wells.
7) Incubate the plates were incubated in a humidified (90% humidity) Liconic incubator at 37 °C without agitation for 48 hours.
8) Dilute Alamar Blue 1:40 in Ca/Mg-free PBS.
9) To all plates, add 5 µl/well of the diluted Alamar Blue to plates to a final dilution factor of 1:200.
10) Incubate the plates for 2 hours.
11) Read the Relative Fluorescence Intensity (RFU) of wells on a standard plate reader as a measure of relative fungal growth. Envision (Perkin Elmer) plate reader set-up: Ex 544 nm, Em 590 nm, Bandwidth 12 nm, Top read.

Counterscreen Mammalian Cell Toxicity Dose Response (AID Nos. 2327, 488809, 493099, 493147)

Materials and Reagents:

Clear, flat bottom, black 384-well plates (Corning Catalog no. 3712BC Lot no. 35808016); Geldanamycin (AG Scientific Catalog no. G-1047) 15 mM stock solution in DMSO; Fluconazole (Sequoia Research Ltd.) 2 mg/ml stock solution in PBS; Alamar Blue (AG Scientific Catalog no. DAL1100, Lot no. 151016SA); PBS w/o Calcium and Magnesium (Cellgro Catalog no. 21-040-CV)

Assay Medium

Optimem medium (Invitrogen Catalog no. 31985-070; Lot no. 548536); 2.5% (v/v) Fetal Bovine Serum (Hyclone Catalog no.30071.03; Lot no. ARF26748); 1% (v/v) Pen/Strep solution (Invitrogen Catalog no.15140-122; Lot no. 529891)

Cell Inoculum
Test Strain: NIH-3T3 mammalian fibroblasts (ATCC CRL No. 1658)

1) Plate cells in 384-well plates at 6,000 cells/well in 20 µl assay medium.
2) Incubate plates overnight at 37 °C under 5% CO_2.

Procedures:

1) After overnight culture, pin compounds into wells at 100 nl/well using the CyBio CyBi-Well pinning instrument.
2) After pinning compounds, add 20 µl of assay medium supplemented with fluconazole to each well. To a final nominal concentration of 8 µg/ml fluconazole.
3) Return the plates to the tissue culture incubator and incubate the culture for an additional 48 hours at 37 °C under 5% CO_2.
4) At the completion of this incubation, add Alamar Blue solution diluted 1:40 in PBS to each well (10 µl/well) to achieve a final dilution of 1:200.
5) Incubate the plates for an additional 2-3 hours at 37 °C under 5% CO_2.
6) Read the Relative Fluorescence Intensity (RFU) of wells was read on a standard plate reader as a measure of relative cell growth. Envision (Perkin Elmer) plate reader set-up: Ex 544 nm, Em 590 m, Bandwidth12 nm,Top read.

Secondary Single Agent (No Fluconazole) Activity Assay Protocol (AID 488802)

Materials and Reagents:

Clear, flat bottom, black 384-well plates (Corning Catalog no. 3712BC; Lot no. 35808016);Geldanamycin (AG Scientific Catalog no. G-1047) 15 mM stock solution in DMSO; Pen/Strep (Gibco Catalog no. 10378-016; Lot no21040170) 100X in PBS; Fluconazole (Sigma Catalog no. F829-100MG; Lot no. 098K4715) 2 mg/ml stock solution in PBS; Alamar Blue (AG Scientific Catalog no. DAL1100; Lot no.151016SA);PBS w/o Calcium and Magnesium (Cellgro Catalog no. 21-040-CV)

Synthetic Defined Growth Medium

RPMI 1640 medium, (powder without sodium bicarbonate; Invitrogen 31800-089, Lot 648072);Uridine 8 mg/ml in water (Sigma Catgalog no. U3750; Lot no. 028K0760); Glucose 40% (w/v) in water (Sigma Catalog no. G-5400); MOPS Buffer (Sigma Catalog no. M-1254; Lot no. 098K0033)

1) Prepare 1X RPMI medium by dissolving 10.4 g powdered medium in 800 ml water.
2) Add 34.52 g MOPS. While stirring, adjust pH to 7.0 with 10N NaOH.
3) Add 10 ml uridine solution, 50 ml glucose solution, adjust final volume to 1000 ml. Filter sterilize.

Fungal Inoculum

Test Strain: *C. albicans* CaCi-2 (10)

1) Inoculate 500 µl of yeast from cryopreserved stock into 250 ml shaker flask containing 30 ml growth medium. Shake at 30 °C overnight (16 hours).
2) Read OD 600 of 1 ml of fungal culture in a cuvette using a standard optical density reader (Eppendorf BioPhotometer Plus), with growth medium as a background blank.
3) Dilute to desired volume of fungal inoculum according to following formula: (1/OD measured) X (Desired Final Volume of Inoculum) X 0.3 = Volume of fungal culture (µl) to add to desired volume of growth medium. When added to media in wells, this yields a calculated starting OD of the fungal inoculum of 0.00015.

Procedures:

1) Add Pen/Strep to the media to a final 1% concentration.
2) Use a Thermo Combi nL to dispense 20 µl/well of assay media into all wells.
3) Mix geldanamycin and fluconazole for positive control.
4) Dispense positive control solution into the positive control wells using Thermo Combi nL for a final concentration of 3 µM geldanamycin, and 8 µg/ml fluconazole.
5) Then, pin 100 nl of test compound from compound plates into assay plates using a CyBi-Well pin tool.
6) Dispense 20 µl/well of culture into the assay media in all wells.
7) Incubate the plates in a humidified (90% humidity) Liconic incubator at 37 °C without agitation for 48 hours.
8) Dilute Alamar Blue 1:40 in Ca/Mg-free PBS.
9) To all plates, add 5 µl/well of the diluted Alamar Blue to the plates to a final dilution factor 1:200.
10) Incubate the plates for 2 hours.
11) Read Relative Fluorescence Intensity (RFU) of wells was read on a standard plate reader as measure of relative fungal growth. Envision (Perkin Elmer) plate reader settings: Ex 544 nm, Em 590 nm, Bandwidth 12 nm, Top read.

Hsp90 Binning Protocol

Materials and Reagents:

Corning white 384-well plate (Corning Catalog no. 8867BC, Lot no. 22609019); Radicicol (Sigma Catalog no. R2146);Tropix Gal-Screen (Applied Biosystems Catalog no. T2359, Lot no. 0903044)

Assay media

SD-ADE Yeast nitrogen base w/o ammonium sulfate, minus adenine:
SD Growth Media (MP Biomedical Catalog no. 4027-012; Lot no. 119458); Dextrose 20%;Complete supplement minus adenine (Sunrise Science Catalog no. 1029-100; Lot no. 070409**)**

1) To 100 ml SD Growth Media, add 100 ml 20% dextrose and 780 mg Complete Supplement.
2) Add water to 1 liter. Filter sterilize.

DOC media

SD-ADE (see above); DOC steroid (Sigma Catalog no. D7000)

1) To 100 ml SD-ADE media, add 1 ml DOC.

Cell Inoculum

Test Strain: ATCC 201238 *Saccharomyces cerevisiae* W303 reporter strain

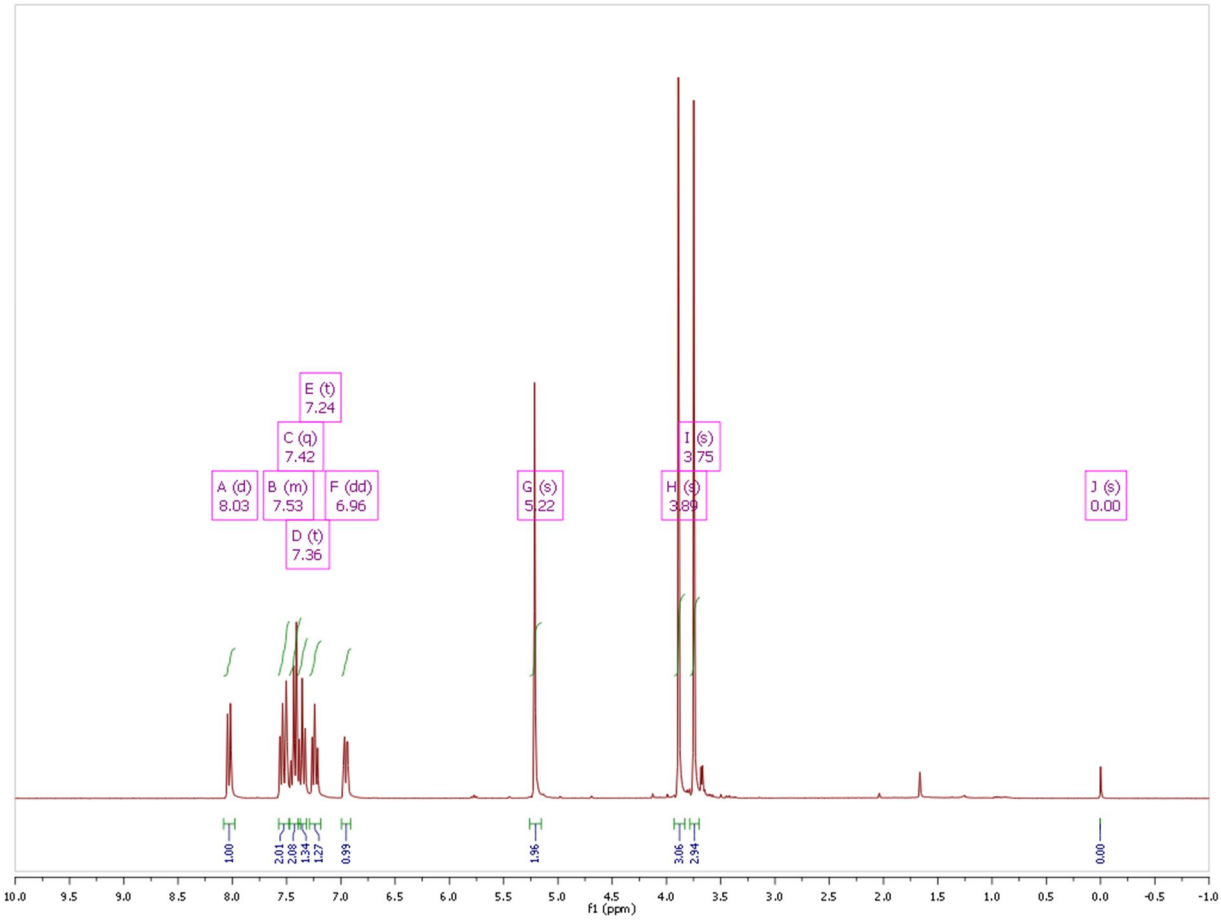
1) Inoculate reporter *Saccharomyces* strain (ATCC Catalog no. 201238) from cryopreserved stock into 250 ml shaker flask containing 20 ml SD-ADE media.
2) Incubate the flask overnight (16 hours) at 37 °C and 150 RPM.

Procedures:

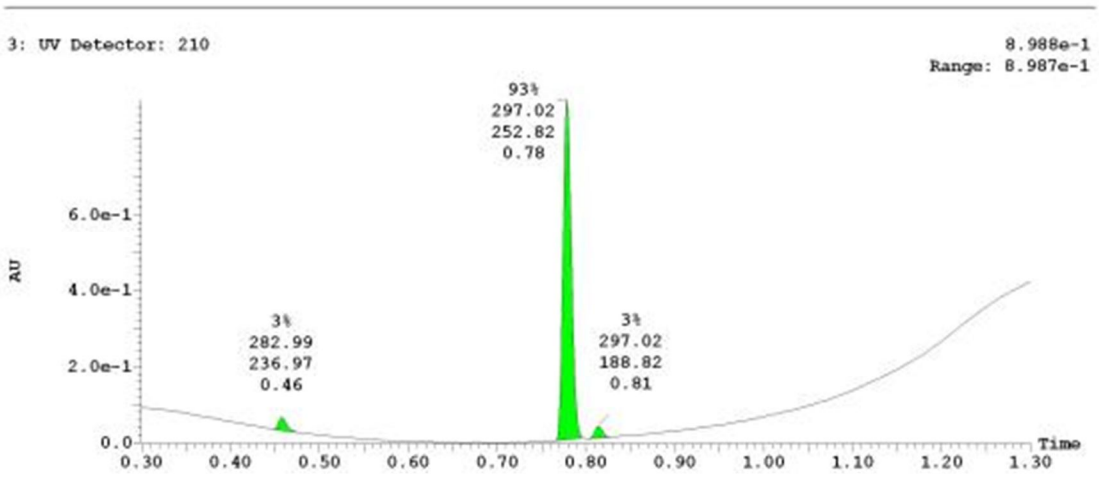
1) Read OD 600 of 1 ml of culture in a cuvette using a standard optical density reader (Eppendorf BioPhotometer Plus), with growth medium as a background blank.
2) Dilute cells to OD = 0.04 in SD-ADE media.
3) To each 384-well white plate, add 20 µl of diluted culture using a Thermo Combi nL.
4) Then, pin 100 nl compounds into plates with a CyBi-Well pin tool.
5) Next, add 5 µM radicicol as a positive control in control wells, dispensing with Thermo Combi nL.
6) With Combi, dispense 20 µl of 20 µM DOC (steroid) in SD-ADE media in pinned plates.
7) Incubate the plates at 30 °C for 75 minutes with agitation.
8) Using Combi, dispense 40 µl Gal-Screen reagent.
9) Incubate the plates at 30 °C for 25 minutes.
10) Read the luminescence of wells on a standard plate reader as measure of relative fungal growth. Envision (Perkin Elmer) plate reader set-up**:** Top read;Luminescence filter (560 nm) at 0.1 second

Appendix C: NMR and LC Data of Probe and Analogs

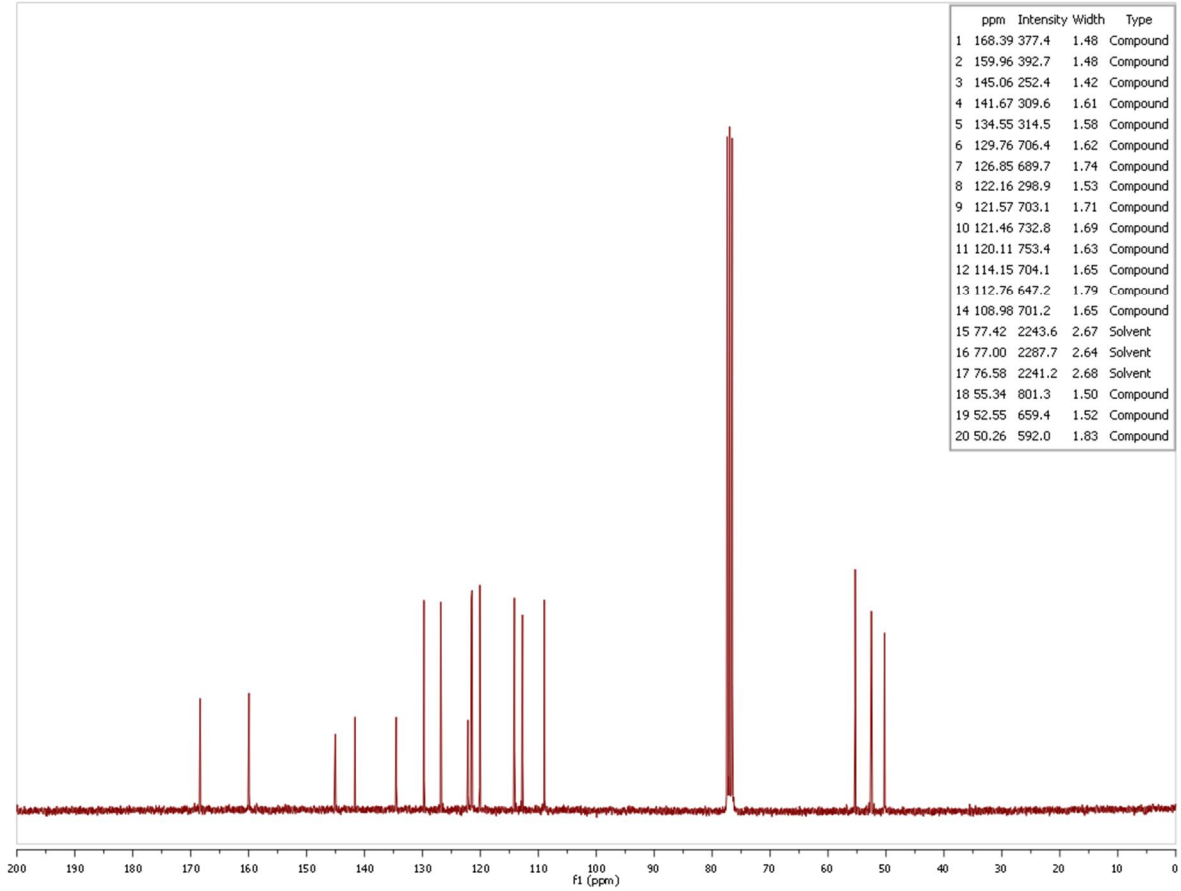
^{1}H NMR (300 MHz, $CDCl_3$) of the Probe (ML212)



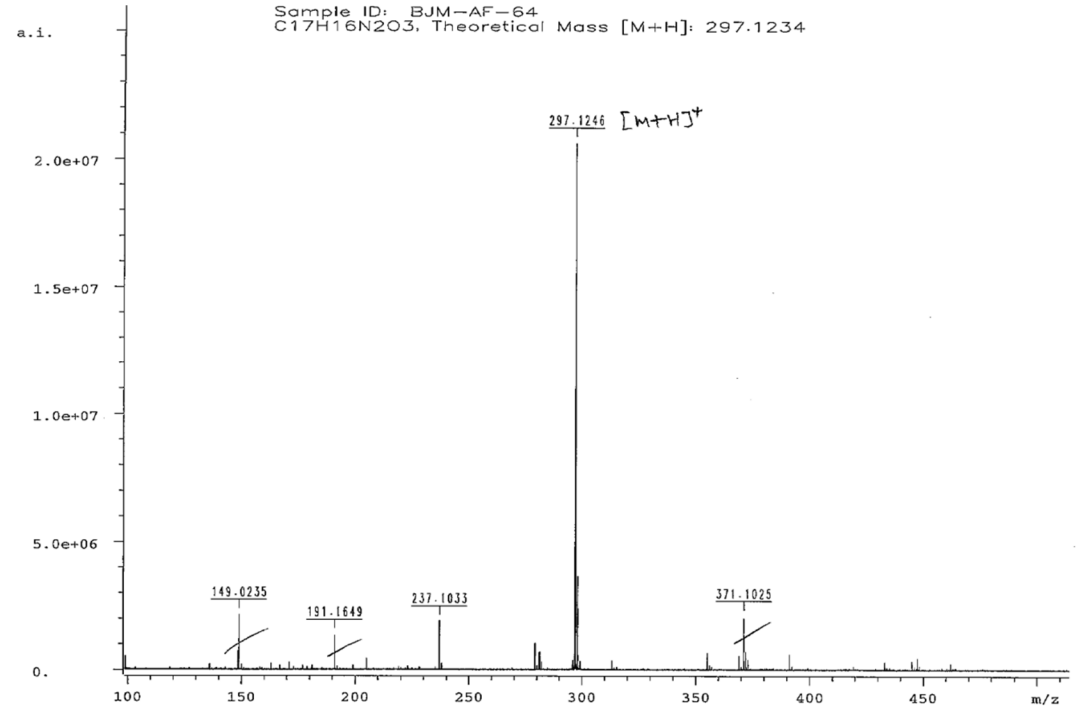
UPLC Chromatogram of the Probe (ML212)



^{13}C NMR (75 MHz, $CDCl_3$) of the Probe (ML212)

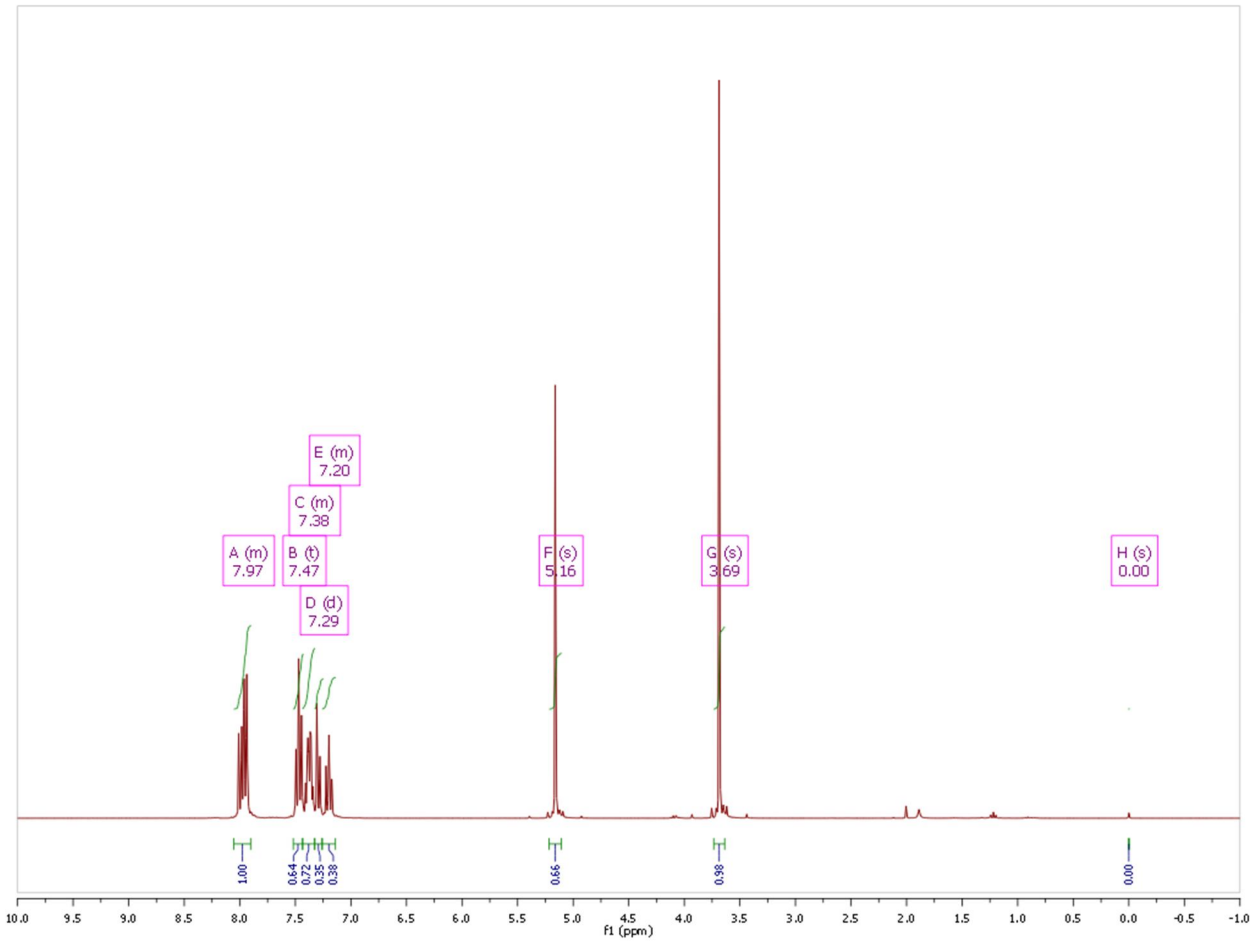


HRMS (ESI) of the Probe (ML212)

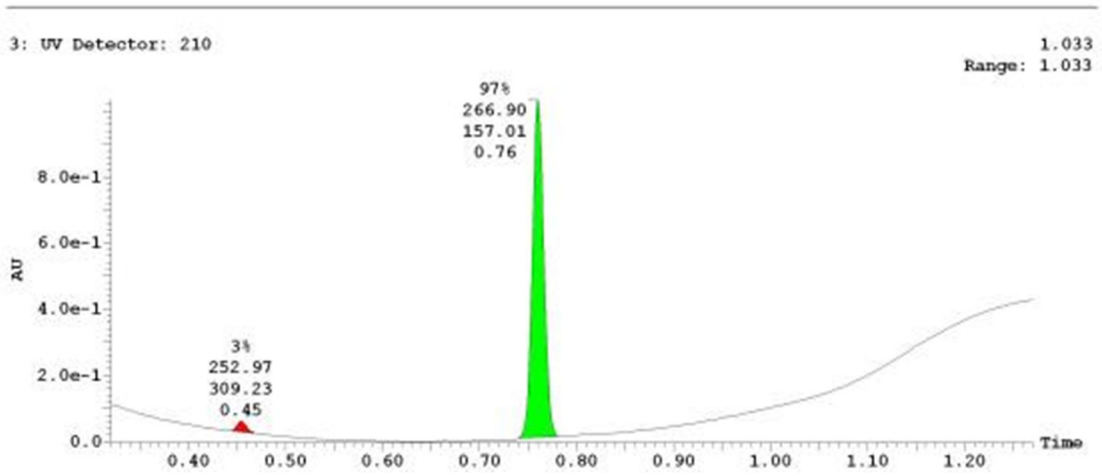


Spectroscopic Data for SAR Analogs

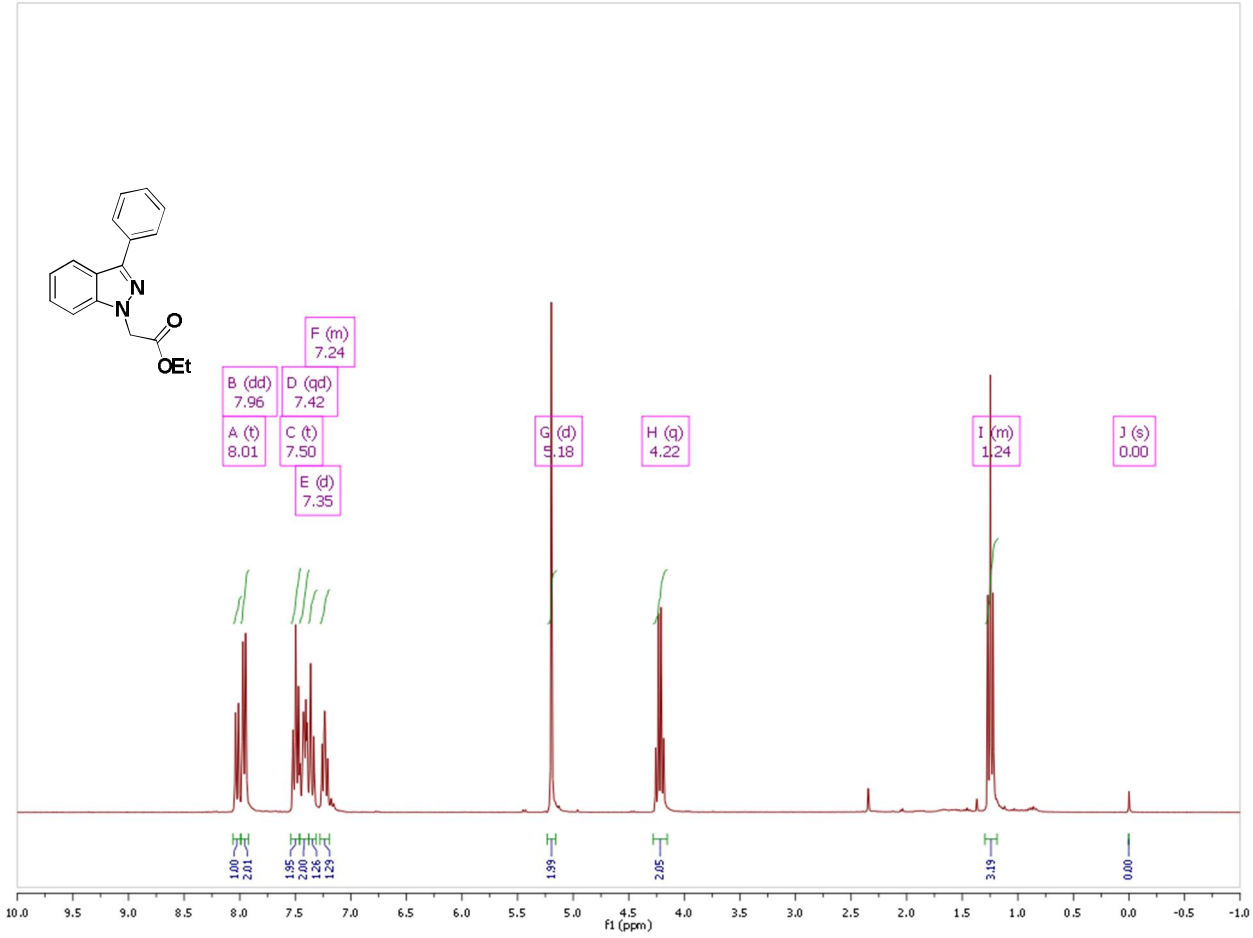
^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 3243873 (Table 3, entry 1)



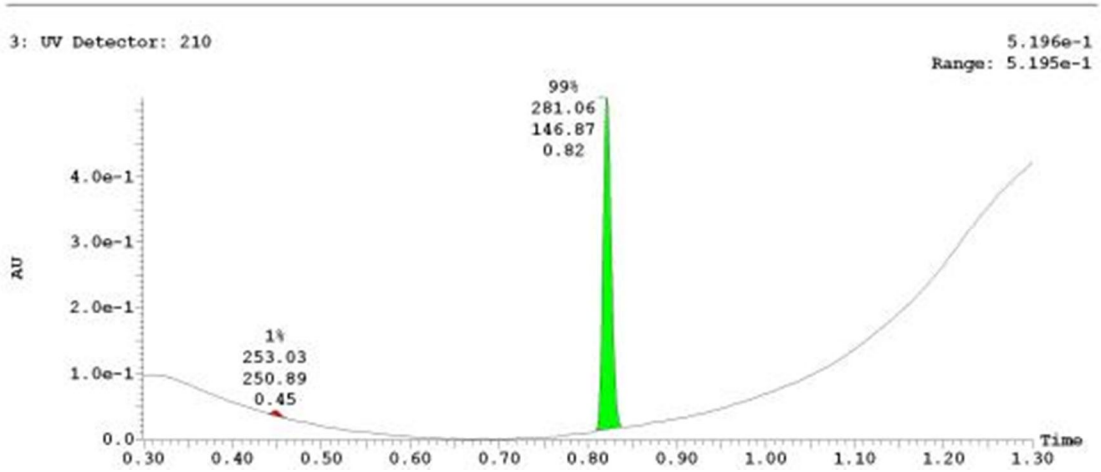
UPLC-MS of Analog CID 3243873



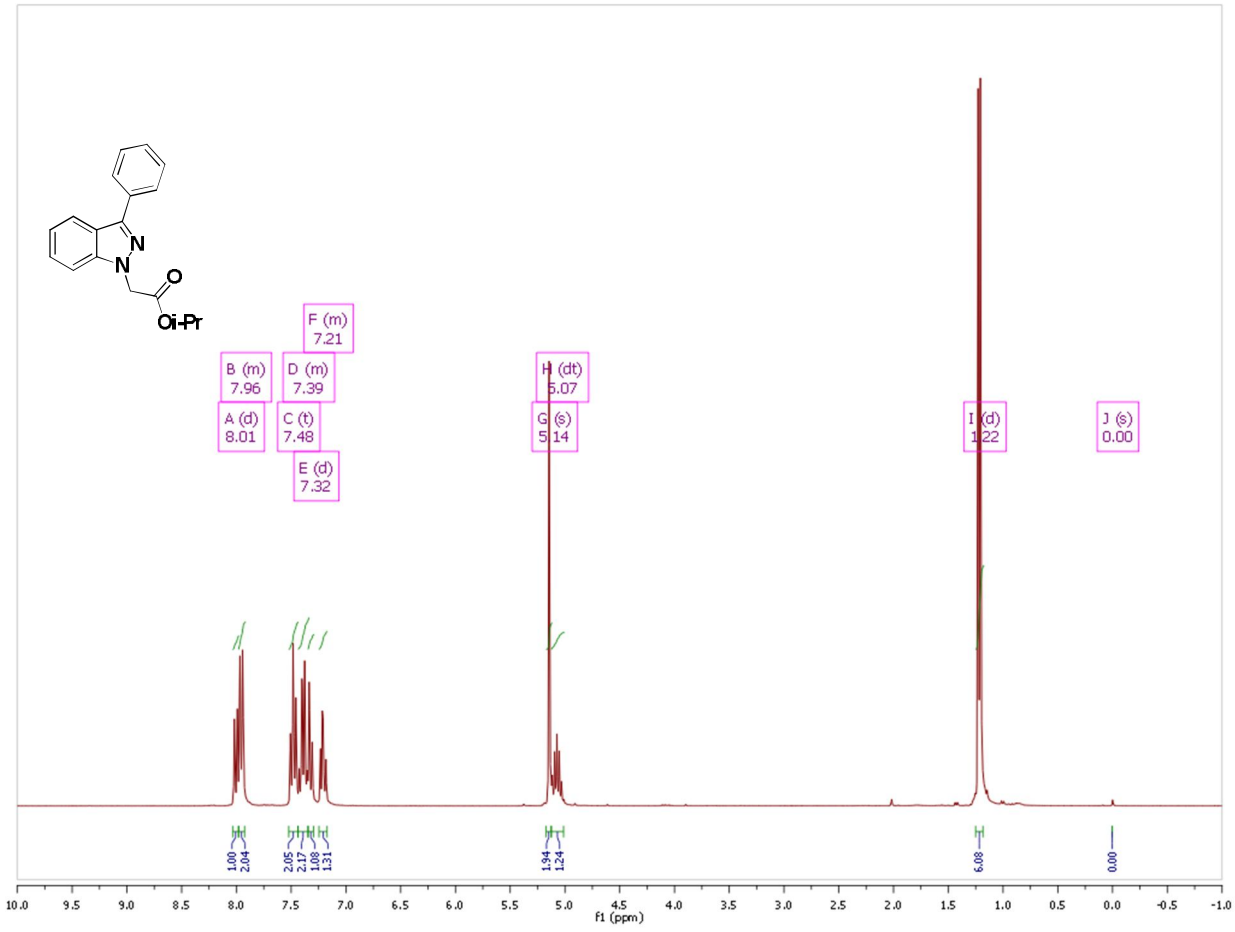
^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 13330959 (Table 3, entry 2)



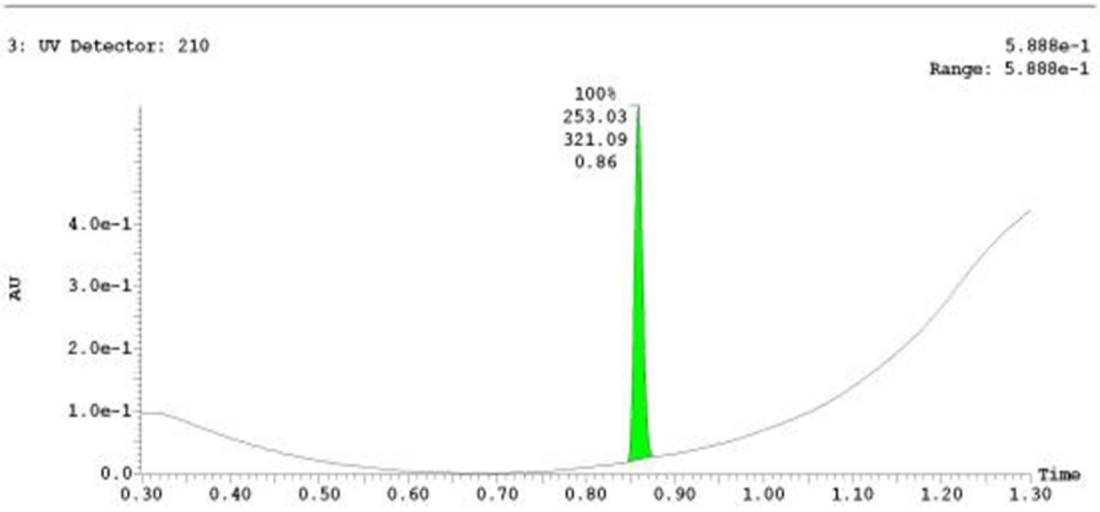
UPLC-MS of Analog CID 13330959



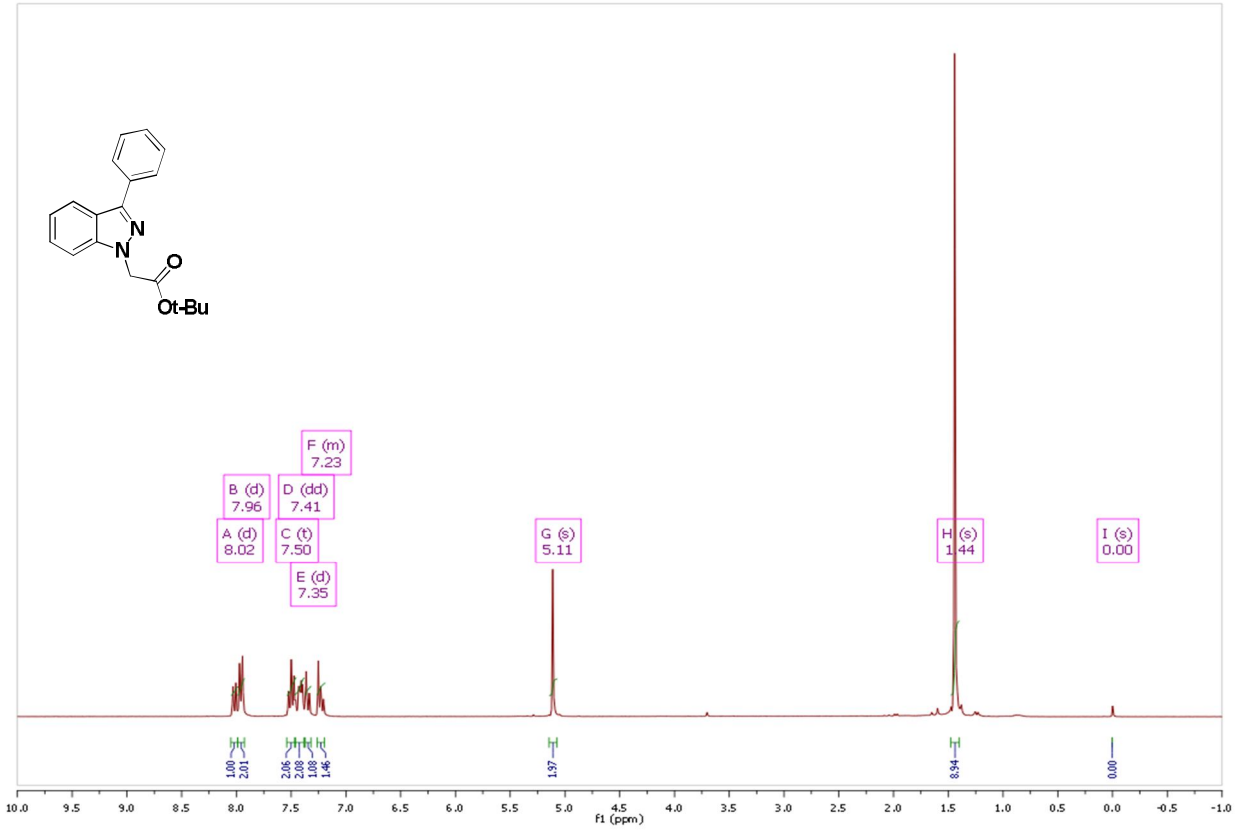
^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 49835820 (Table 3, entry 3)



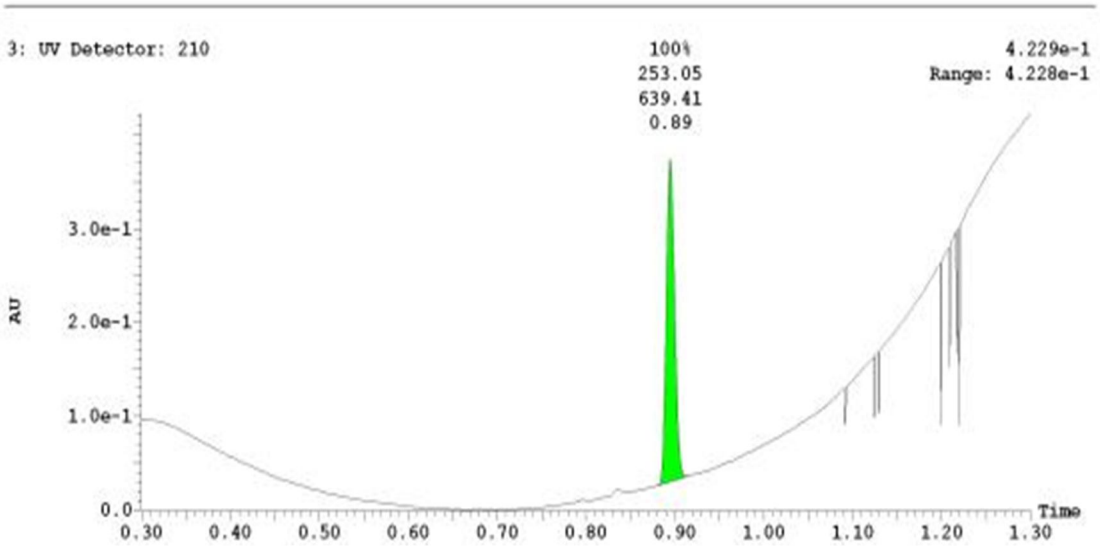
UPLC-MS of Analog CID 49835820



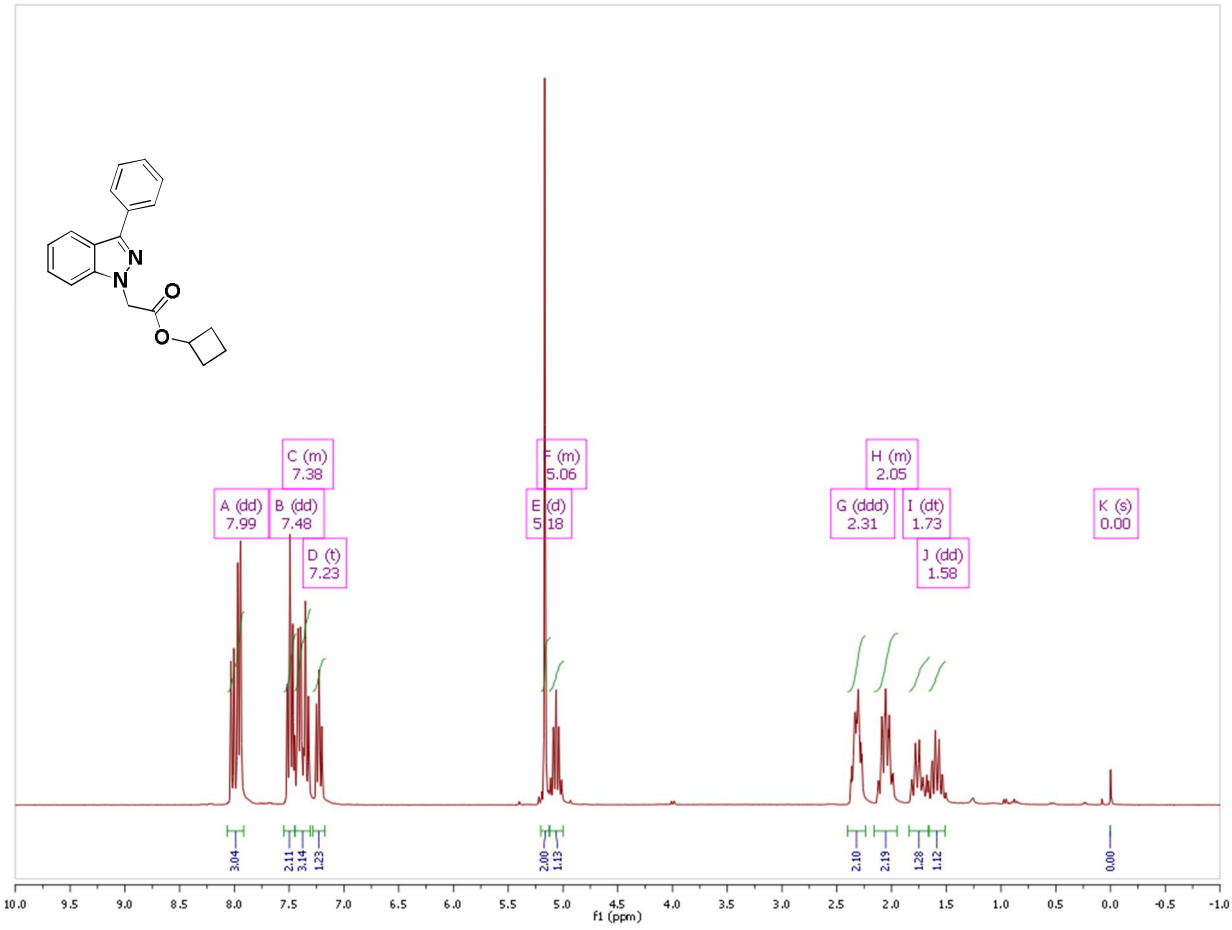
^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 49835839 (Table 3, entry 4)



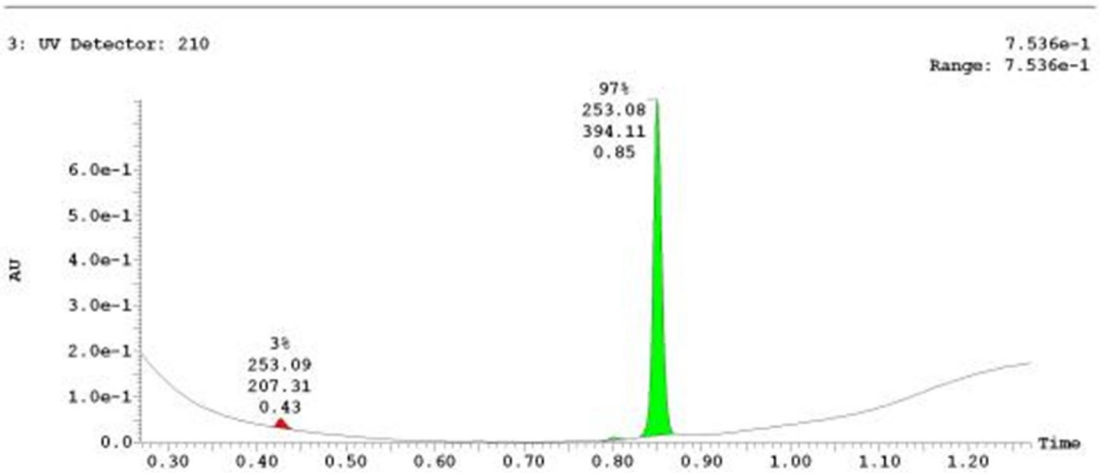
UPLC-MS of Analog CID 49835839



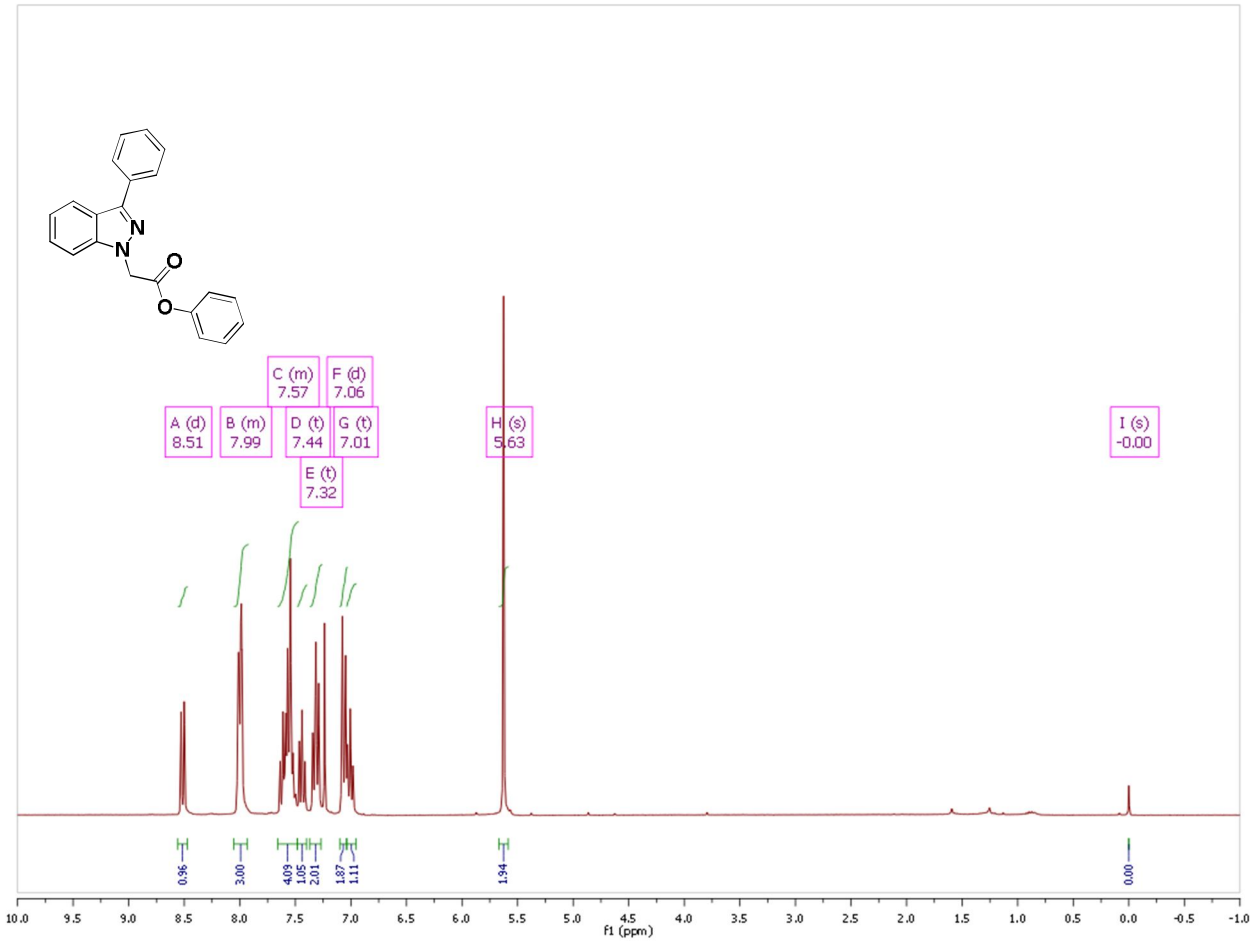
^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 49835870 (Table 3, entry 5)



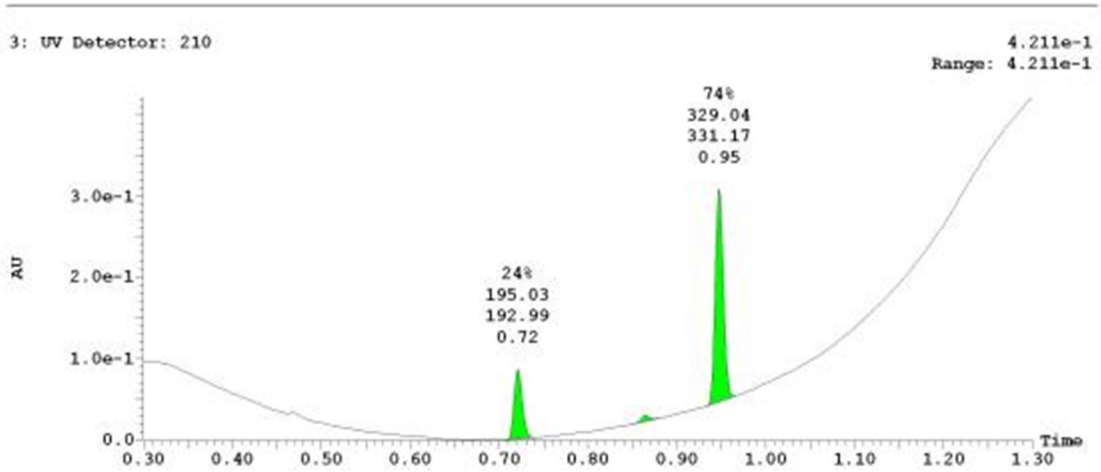
UPLC-MS of Analog CID 49835870



[1]H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 49835857 (Table 3, entry 6)



UPLC-MS of Analog CID 49835857



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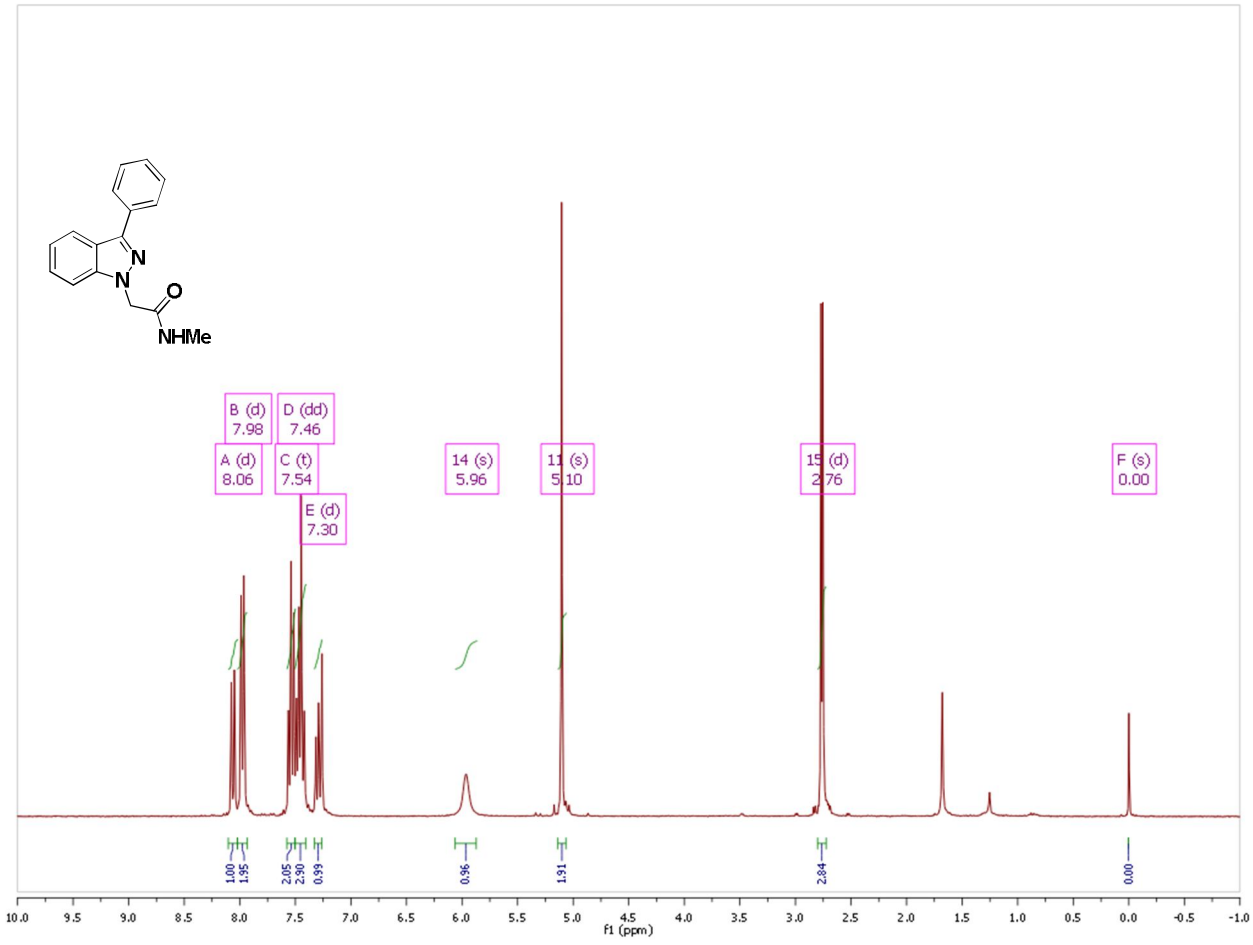
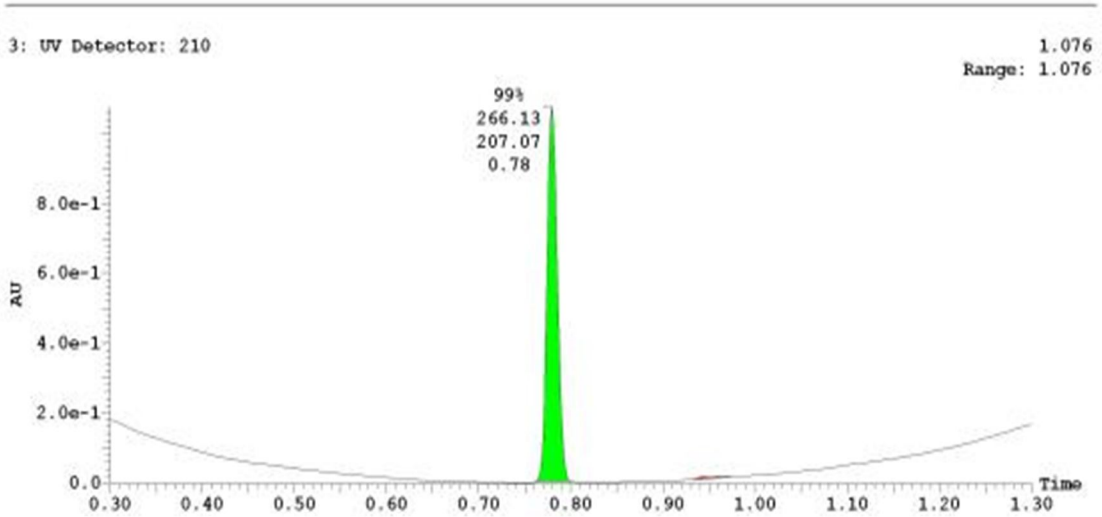
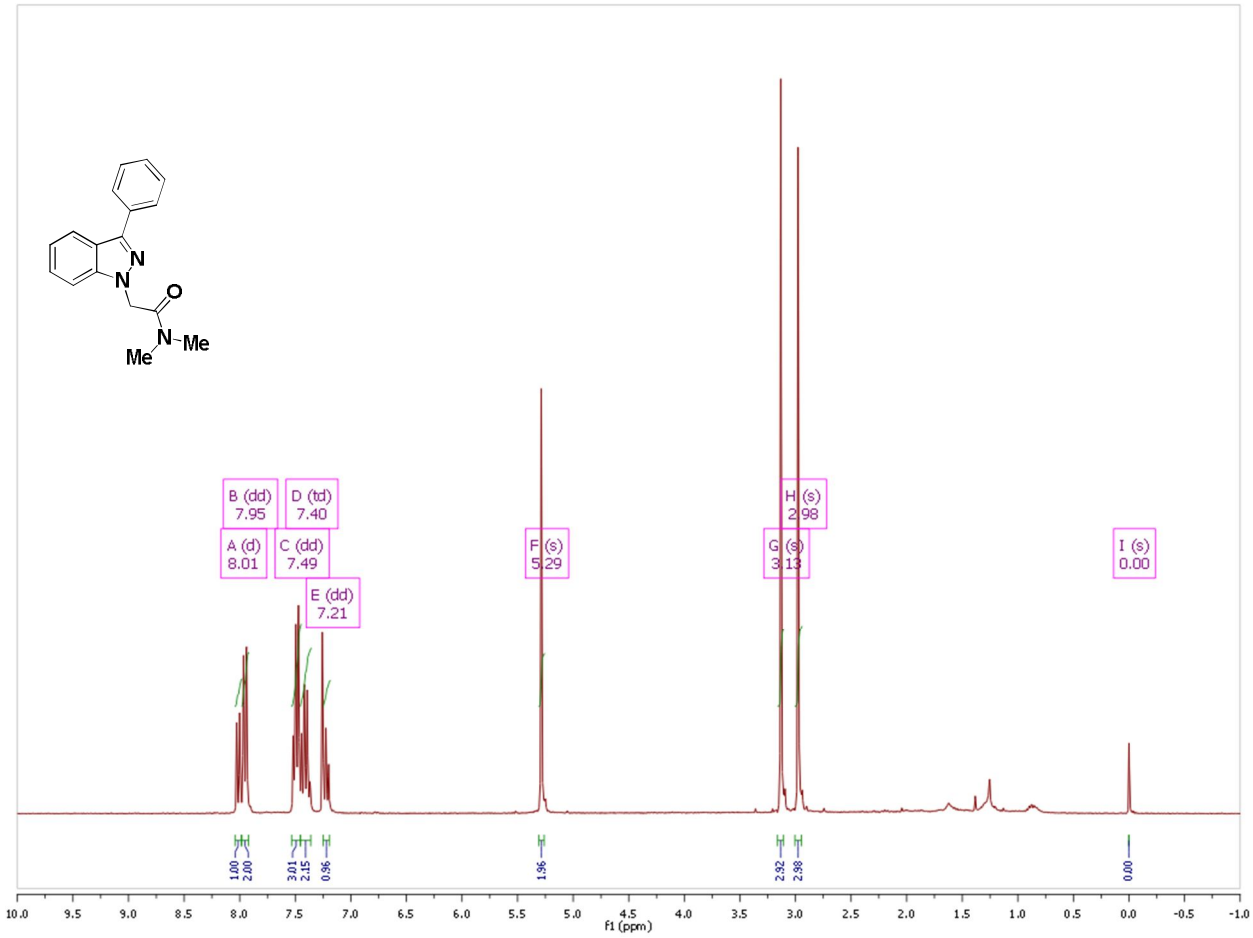


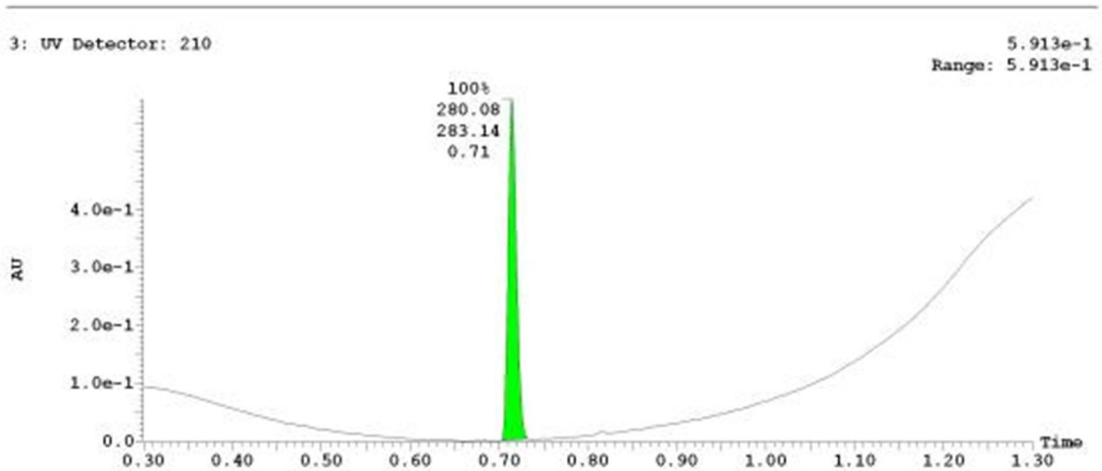
UPLC-MS of Analog CID 13330955



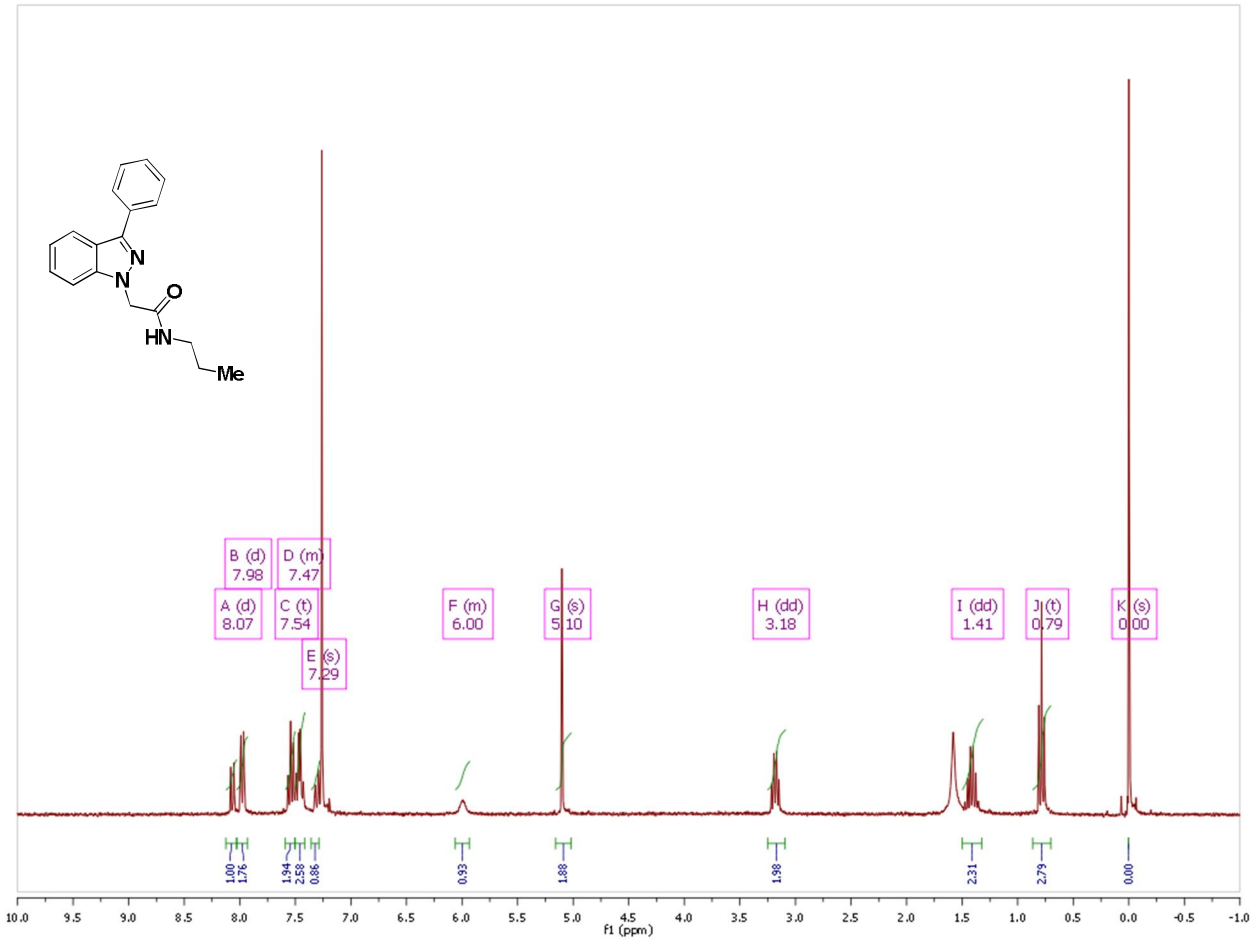
^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 49835882 (Table 3, entry 8)



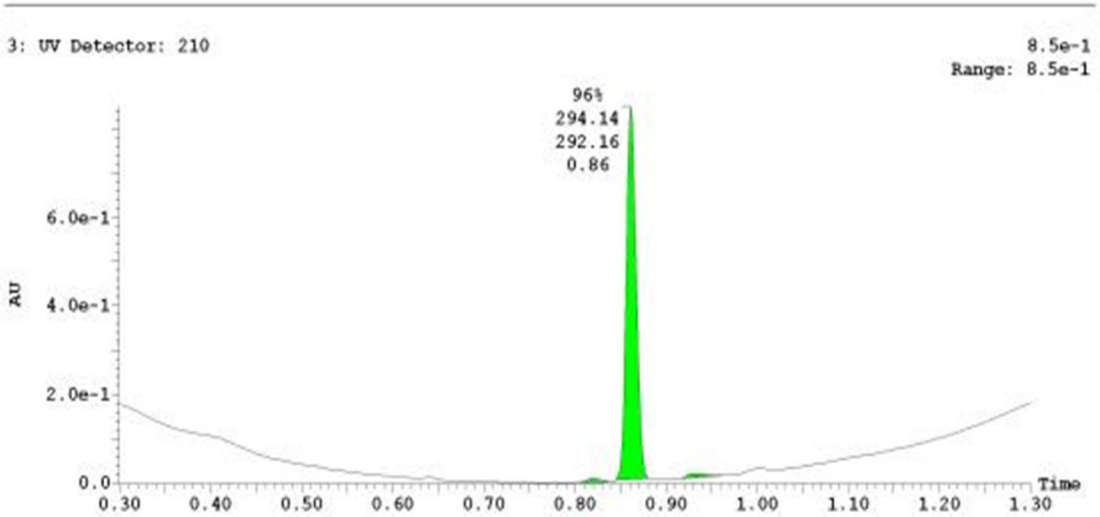
UPLC-MS of Analog CID 49835882



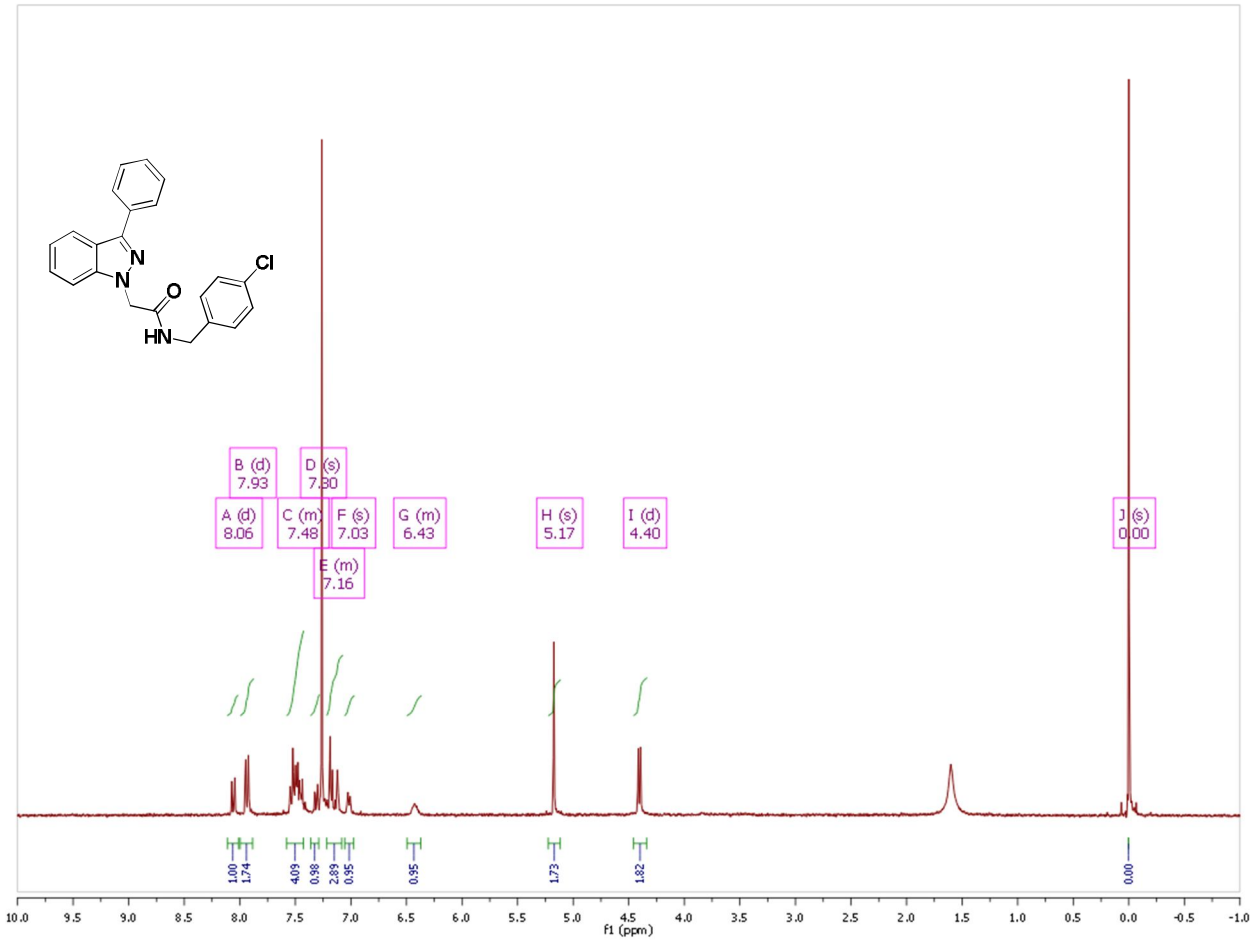
^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 20877577 (Table 3, entry 9)



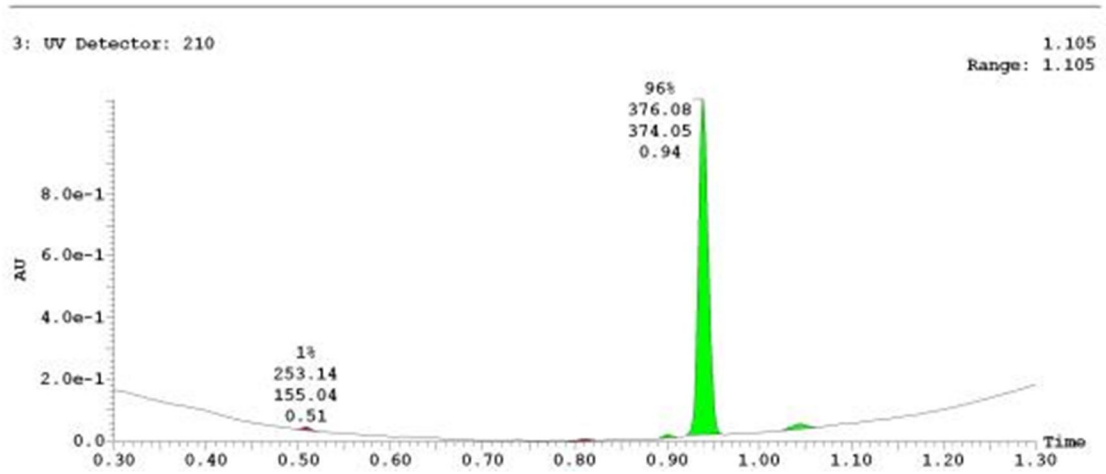
UPLC-MS of Analog CID 20877577



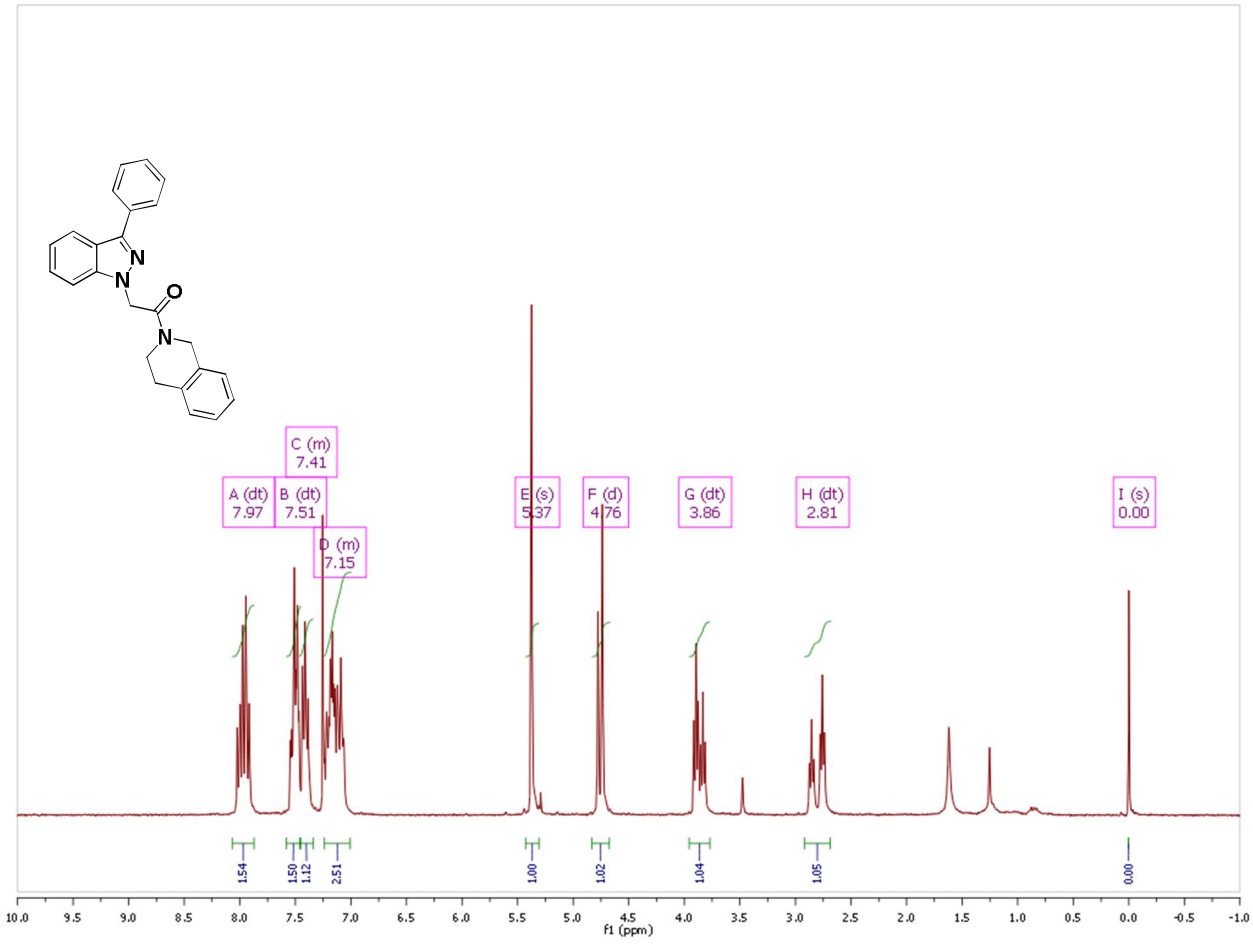
^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 20877568 (Table 3, entry 10)



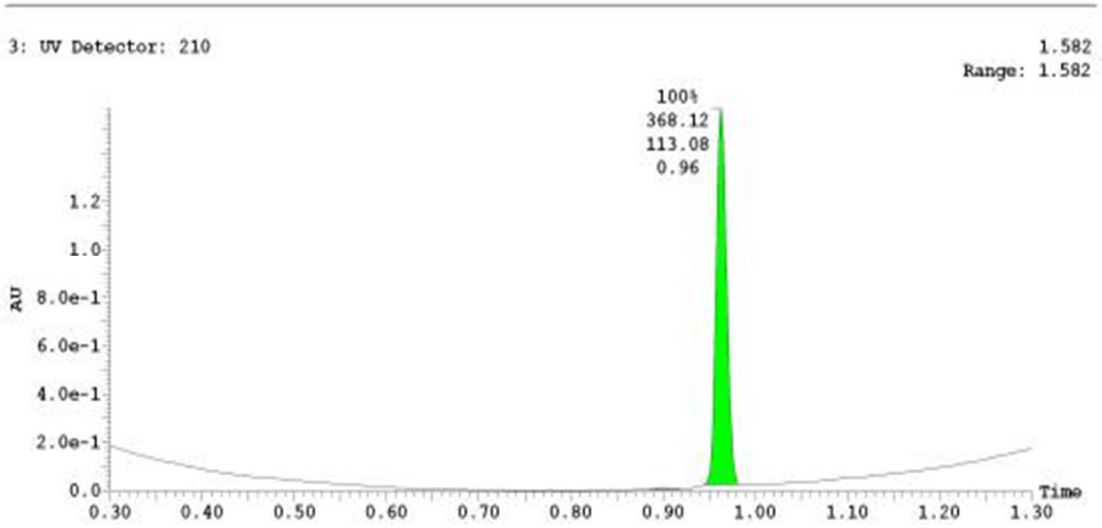
UPLC-MS of Analog CID 20877568



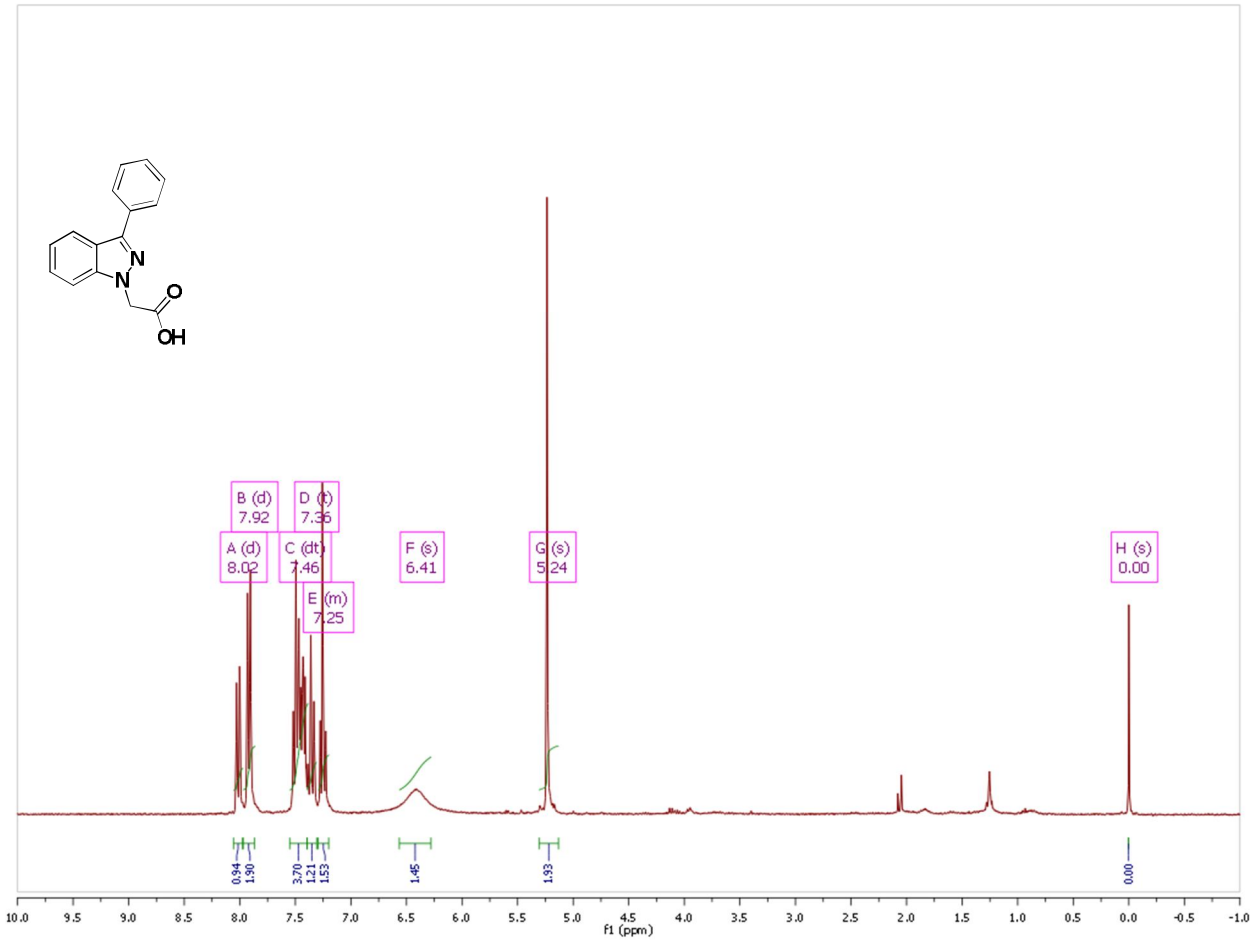
^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 20877557 (Table 3, entry 11)



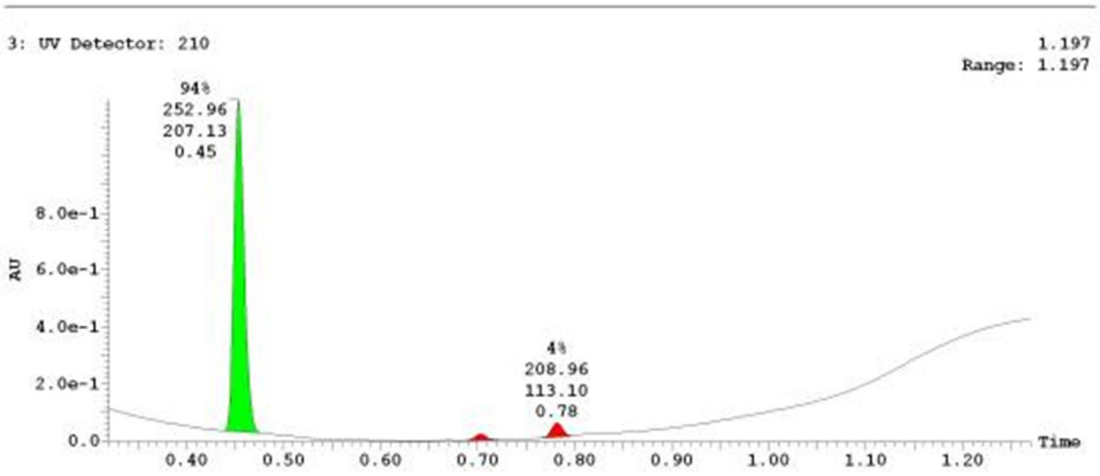
UPLC-MS of Analog CID 20877557



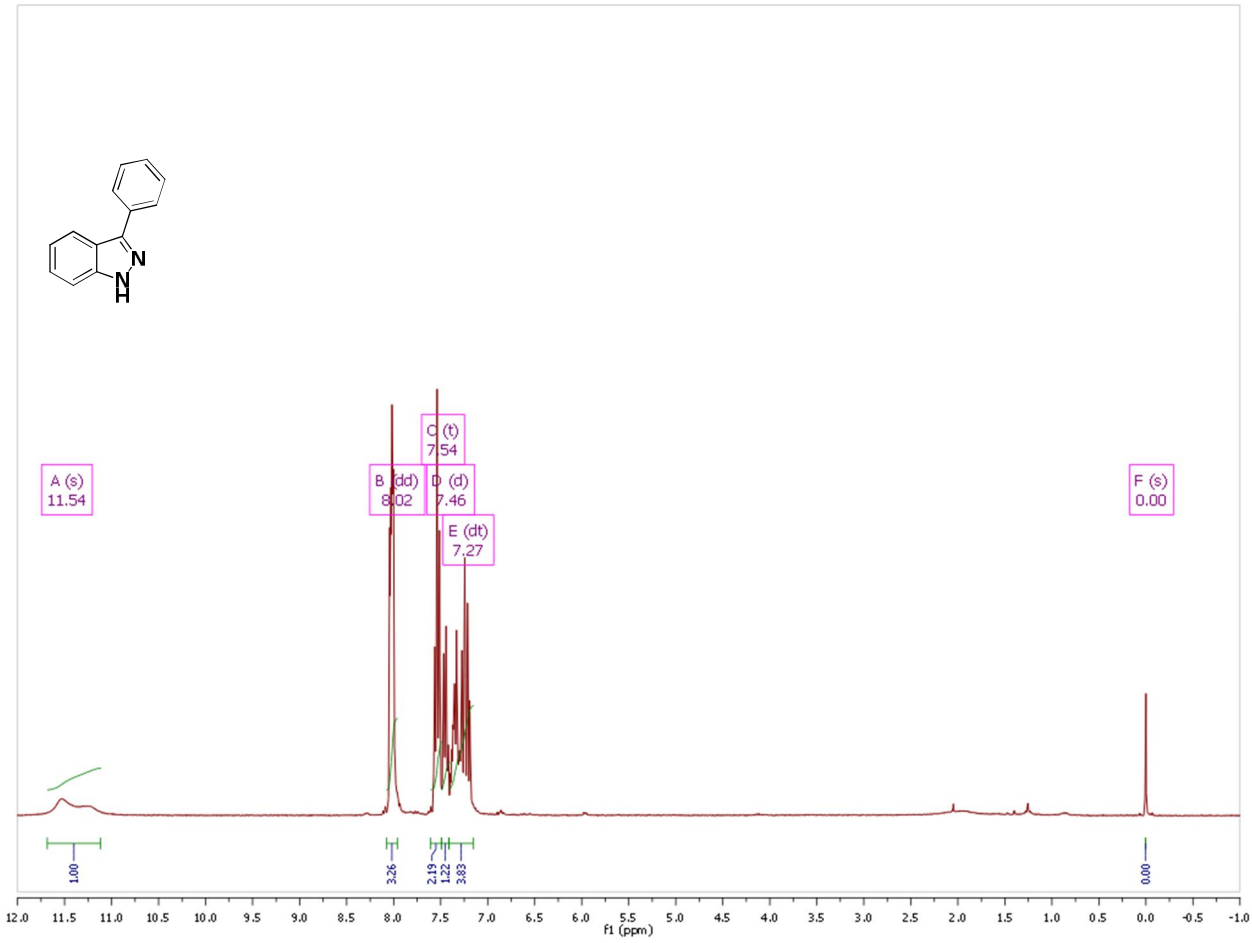
^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 5309553 (Table 3, entry 12)



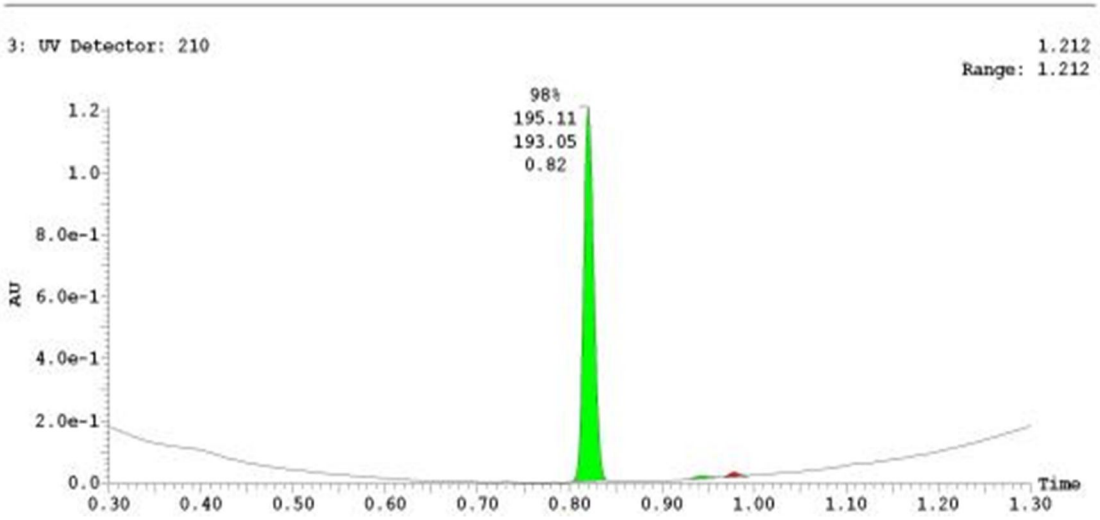
UPLC-MS of Analog CID 5309553



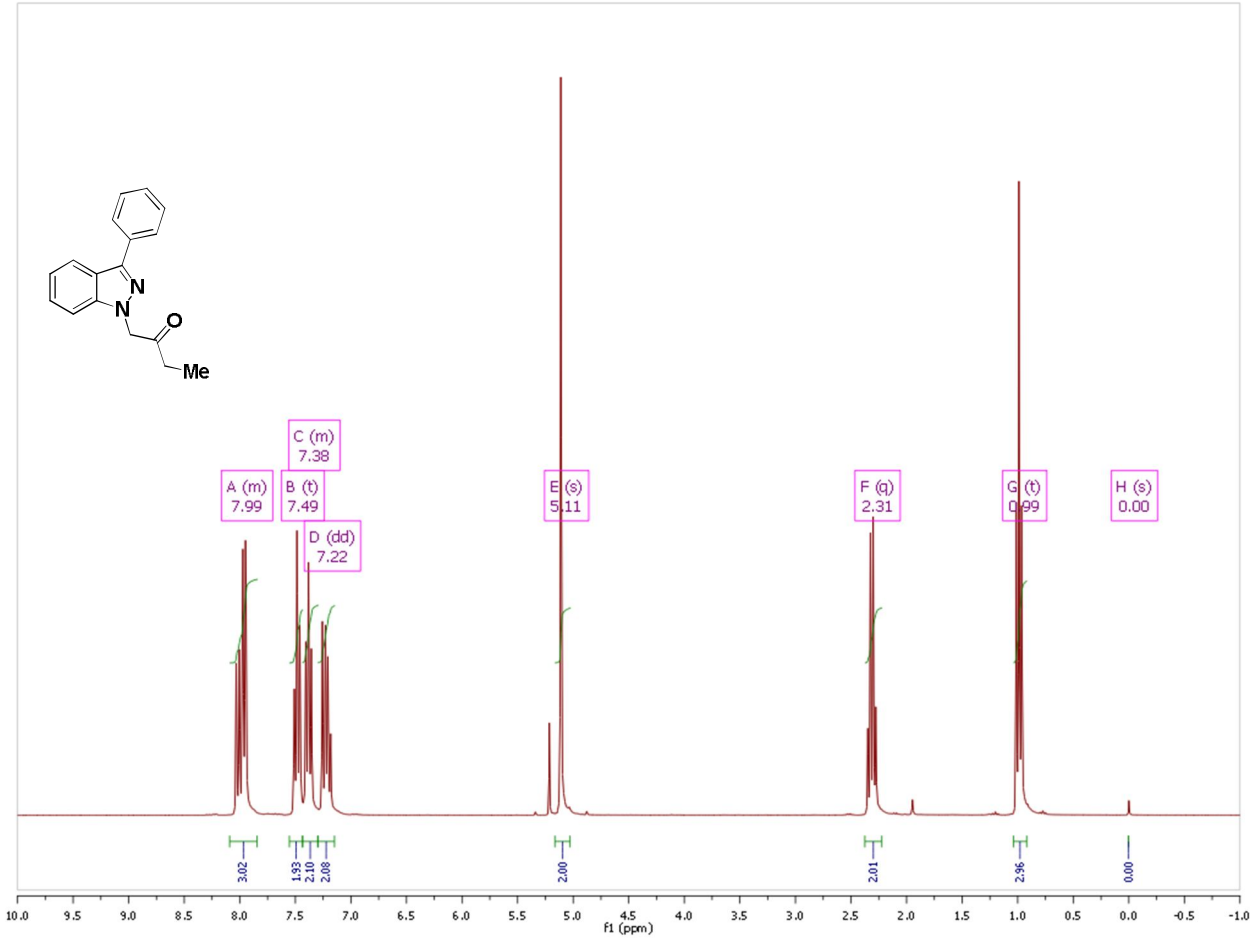
^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 300385 (Table 4, entry 1)



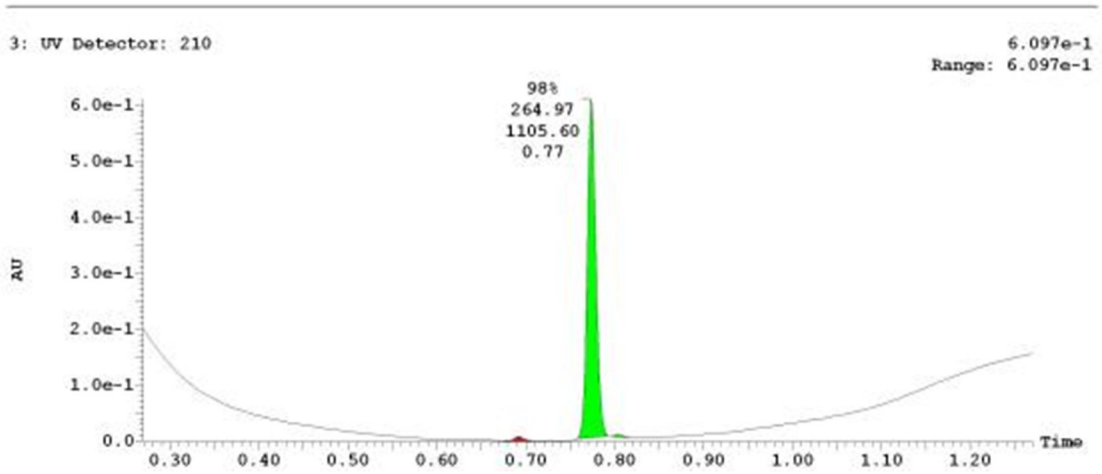
UPLC-MS of Analog CID 300385



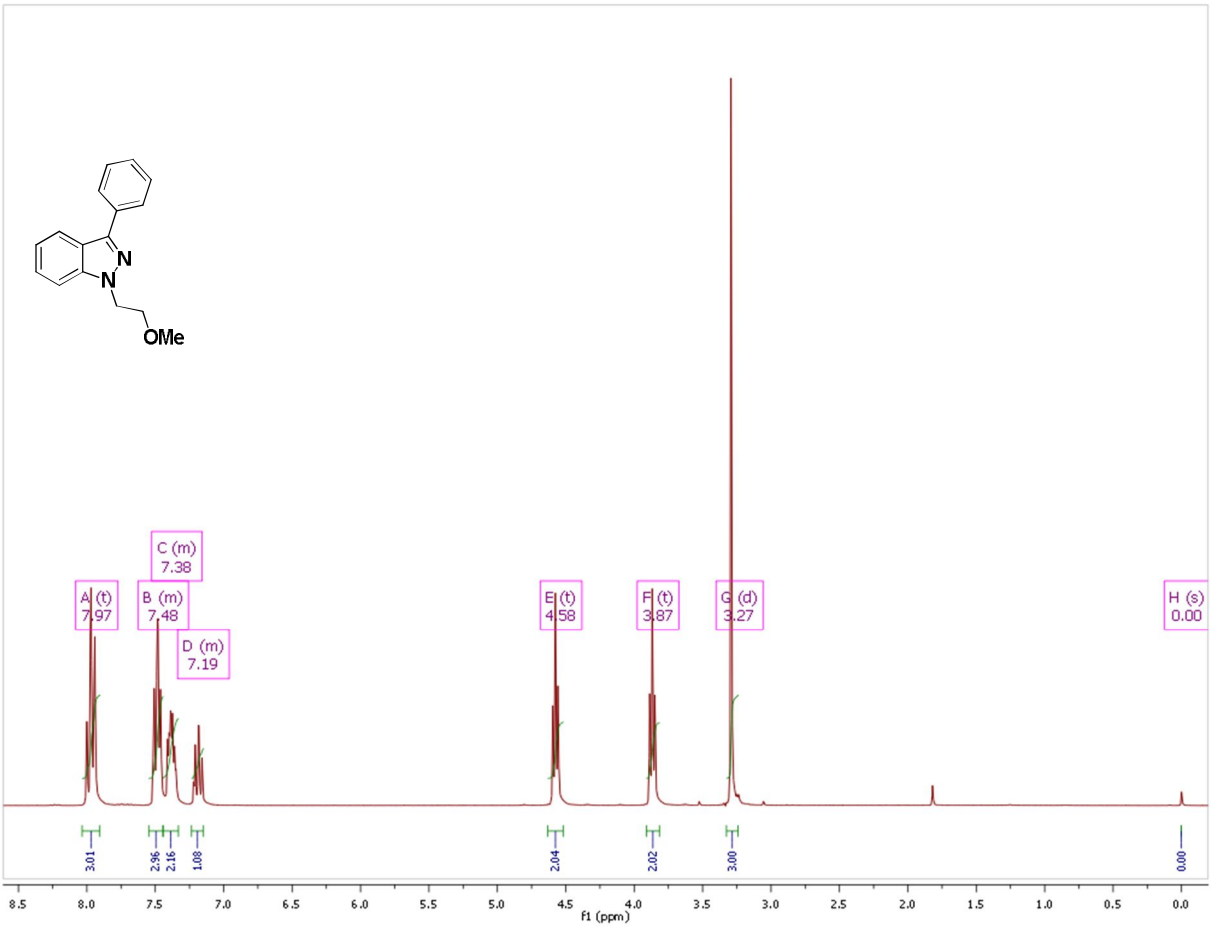
^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 49835816 (Table 4, entry 2)



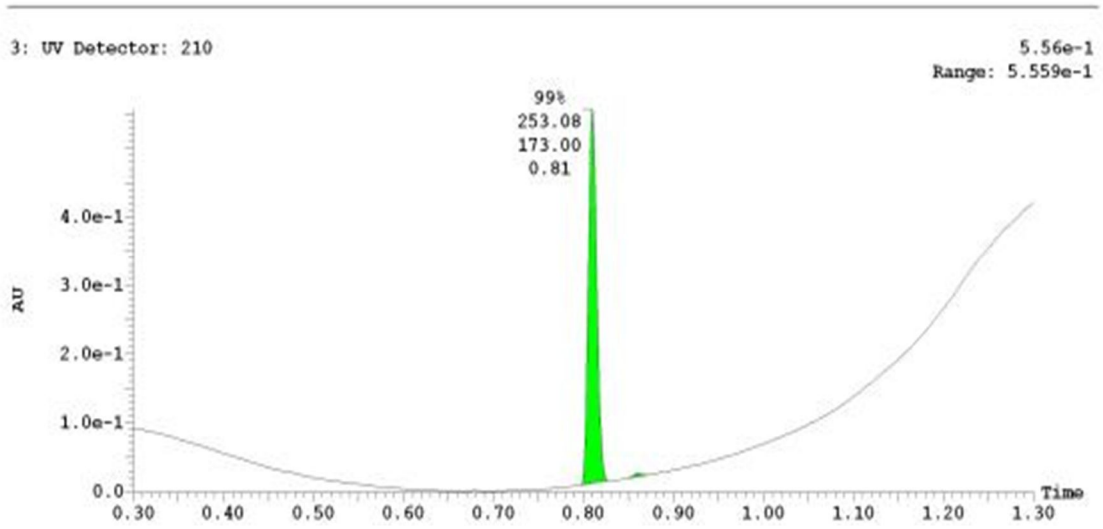
UPLC-MS of Analog CID 49835816



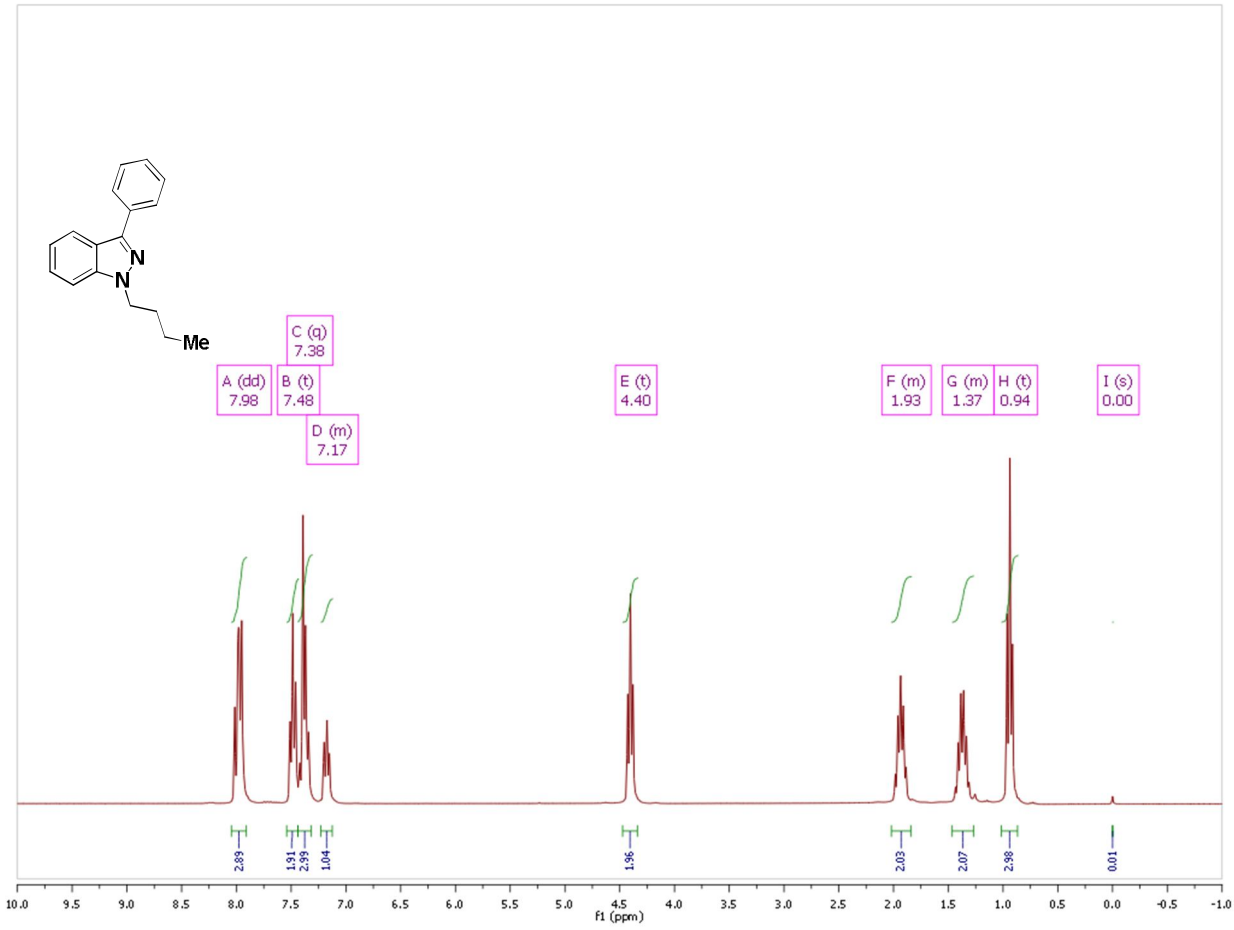
^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 49835825 (Table 4, entry 3)



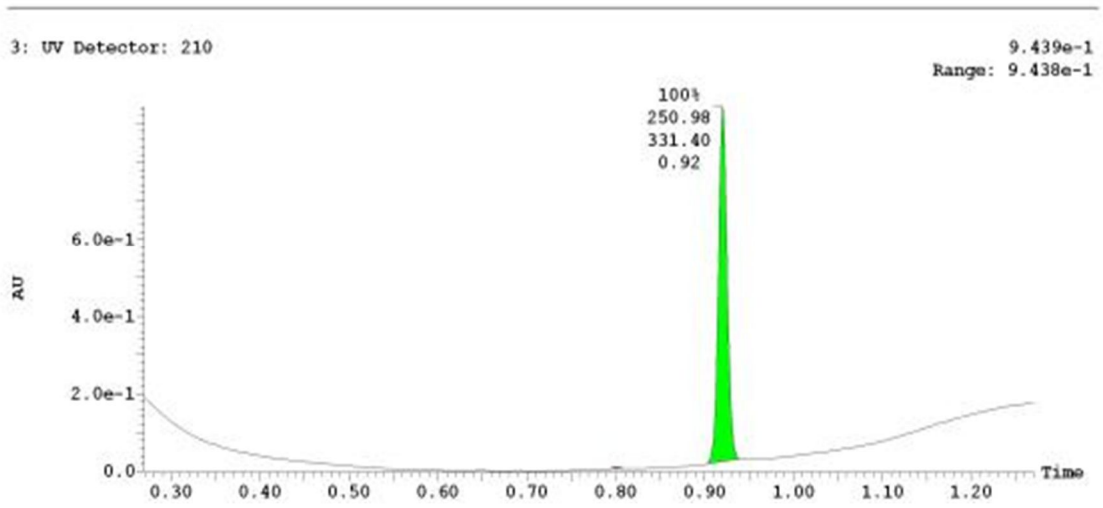
UPLC-MS of Analog CID 49835825



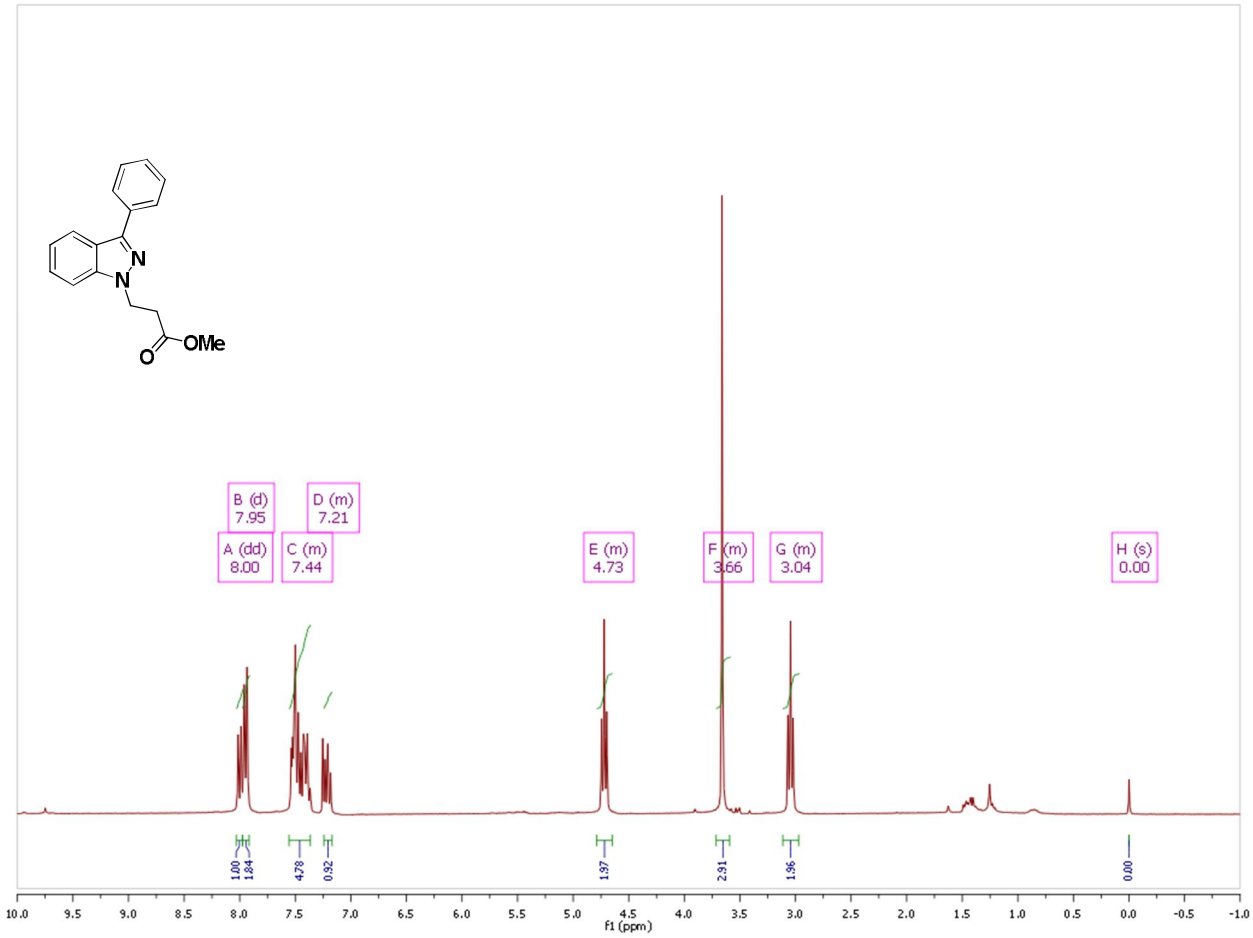
^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 49835848 (Table 4, entry 4)



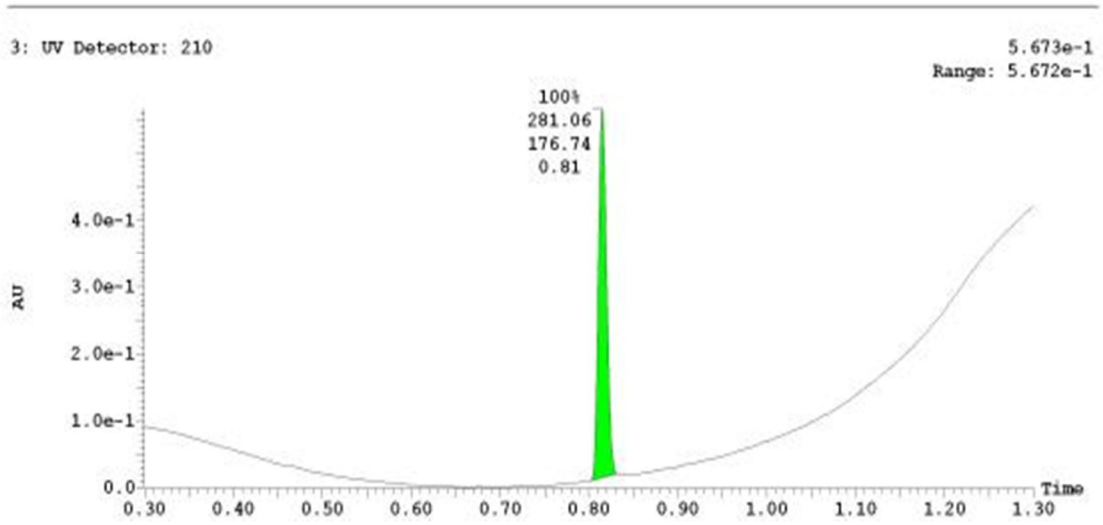
UPLC-MS of Analog CID 49835848



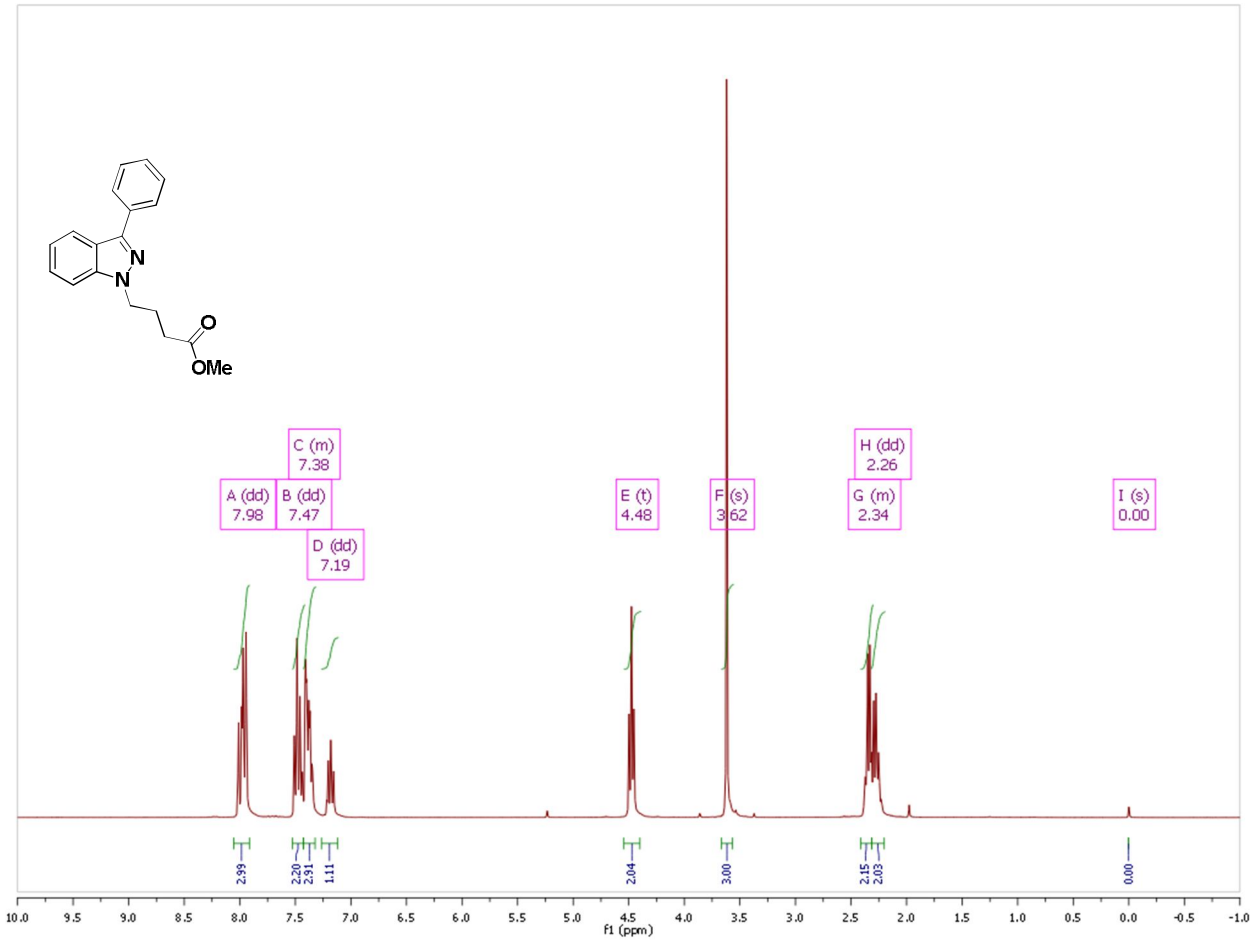
^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 49835835 (Table 4, entry 5)



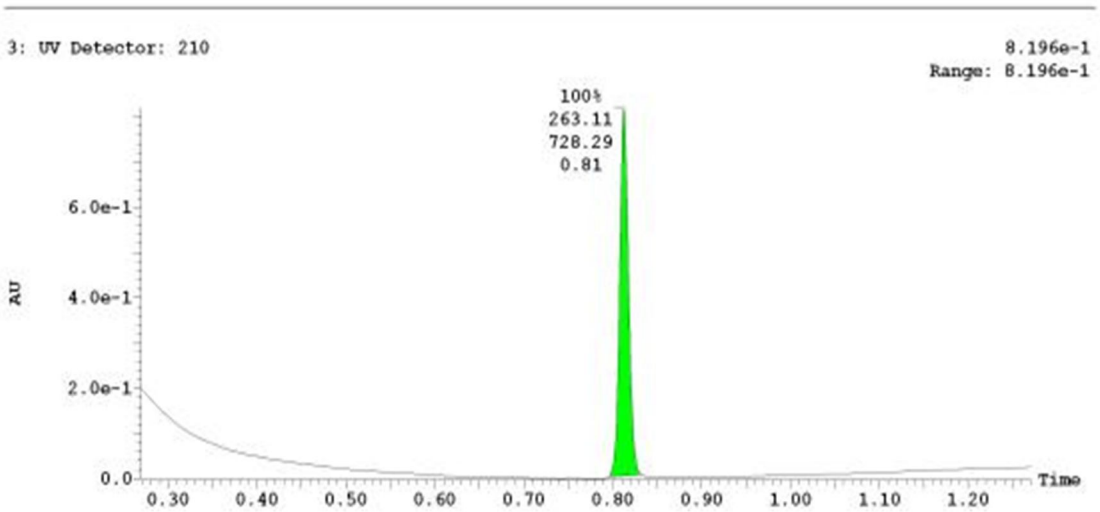
UPLC-MS of Analog CID 49835835



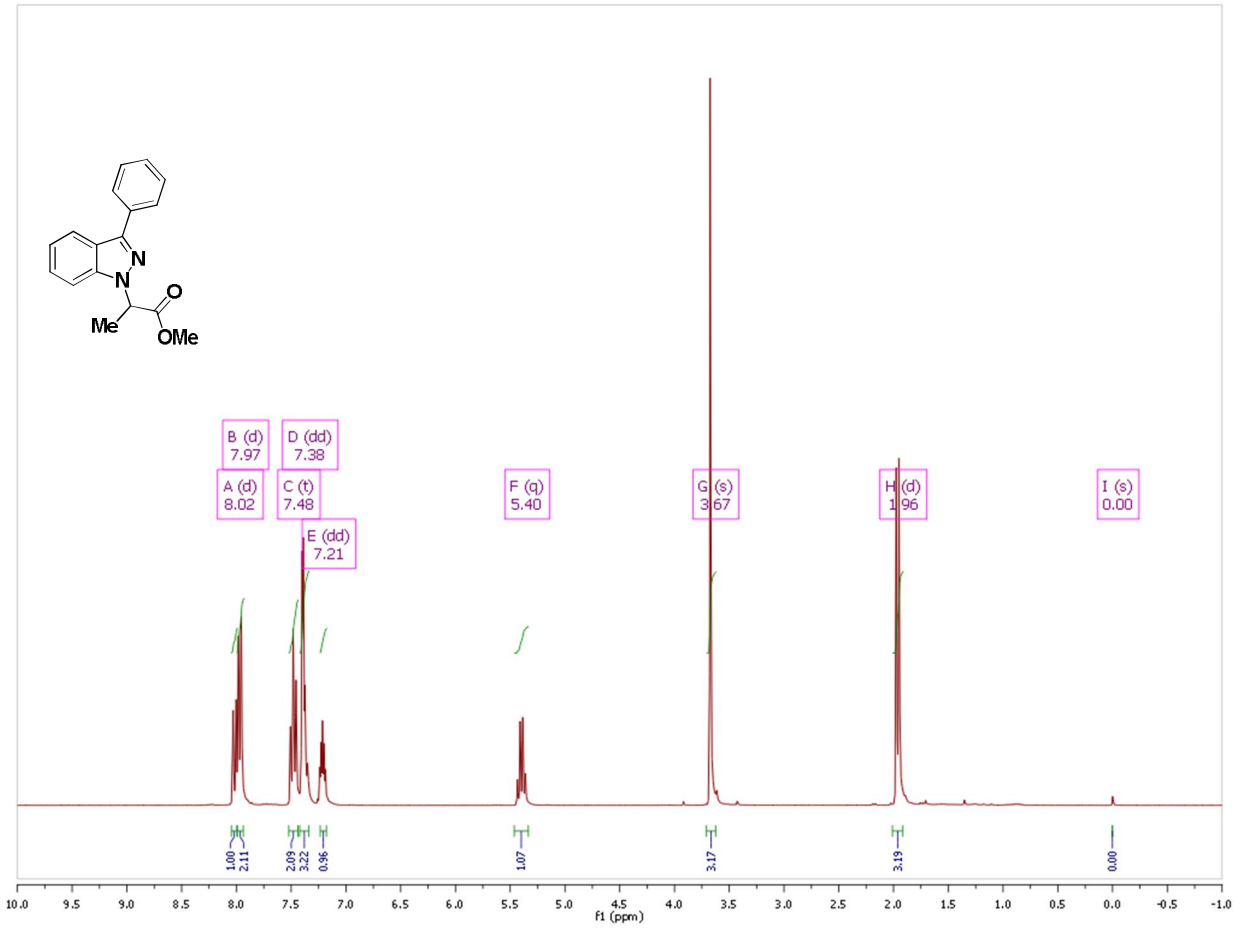
^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 49835881 (Table 4, entry 6)



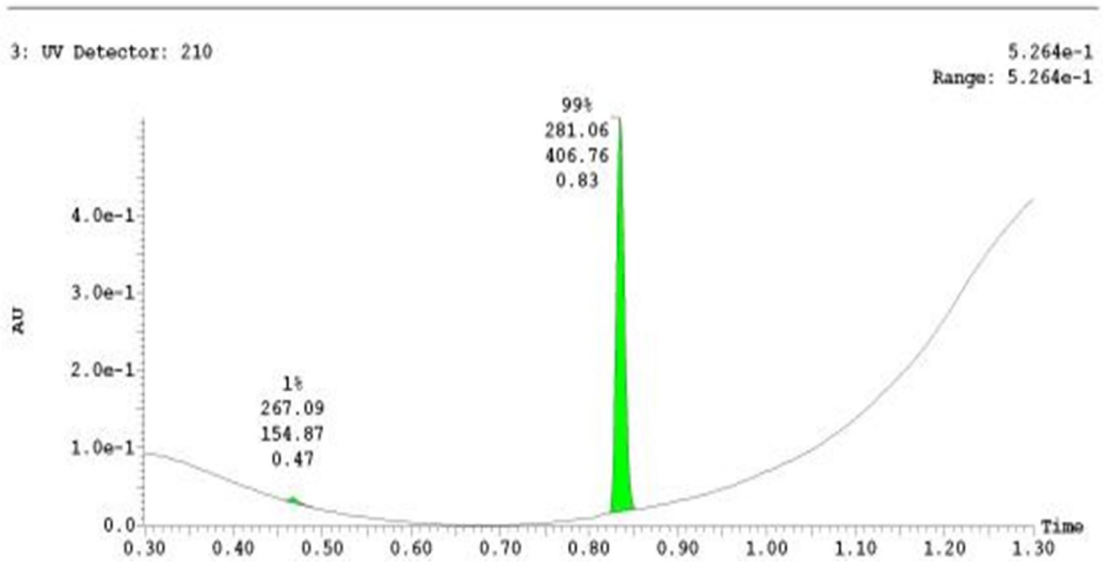
UPLC-MS of Analog CID 49835881



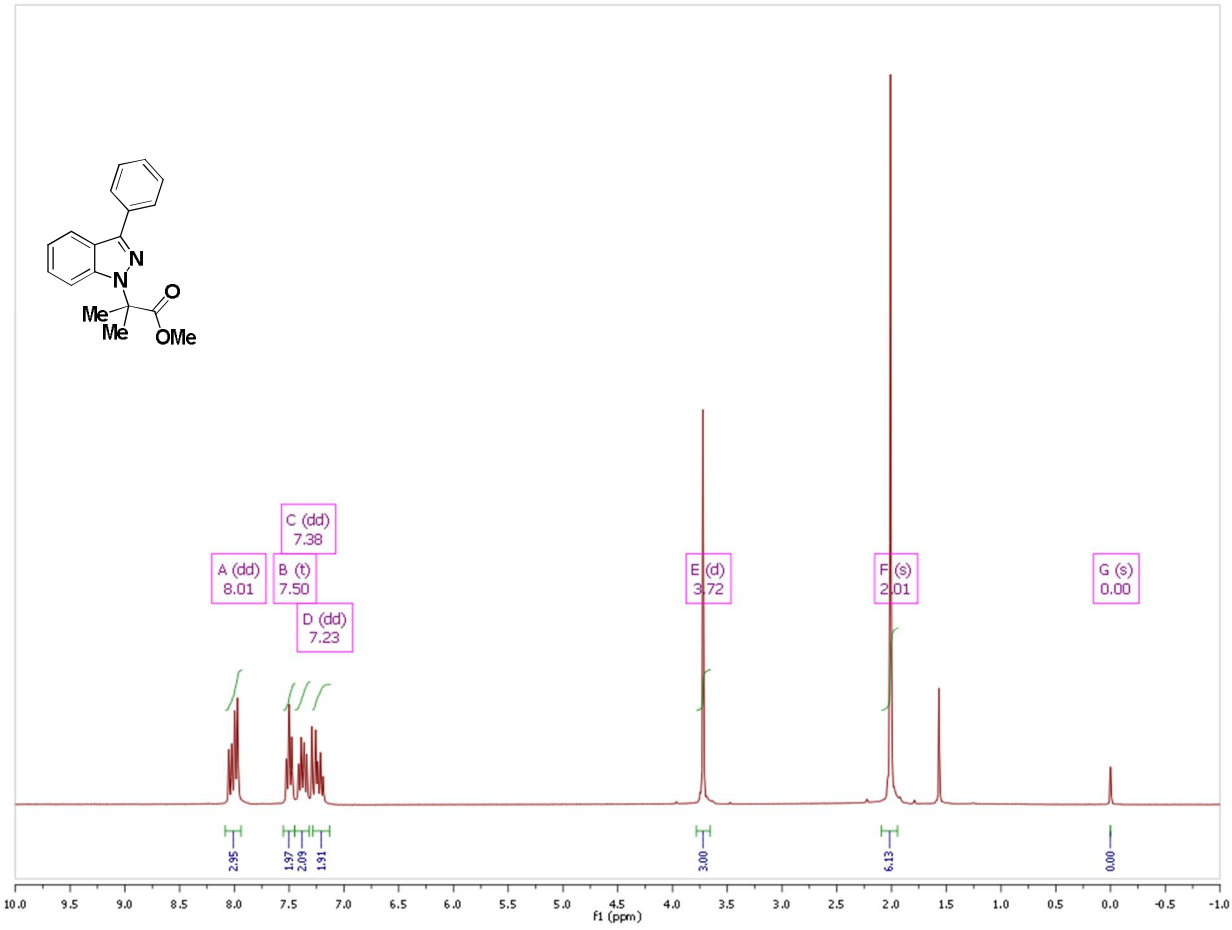
^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 49835813 (Table 4, entry 7)



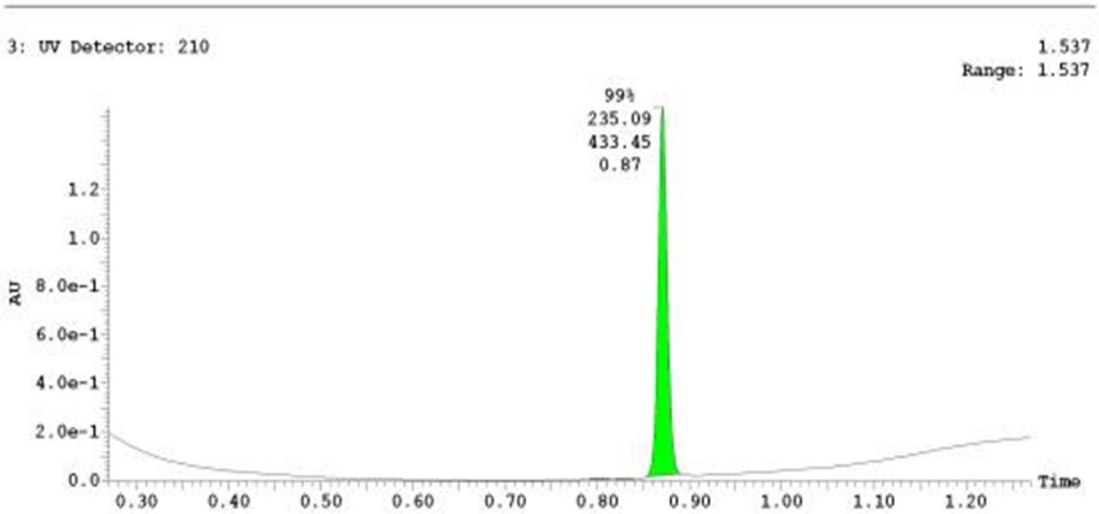
UPLC-MS of Analog CID 49835813



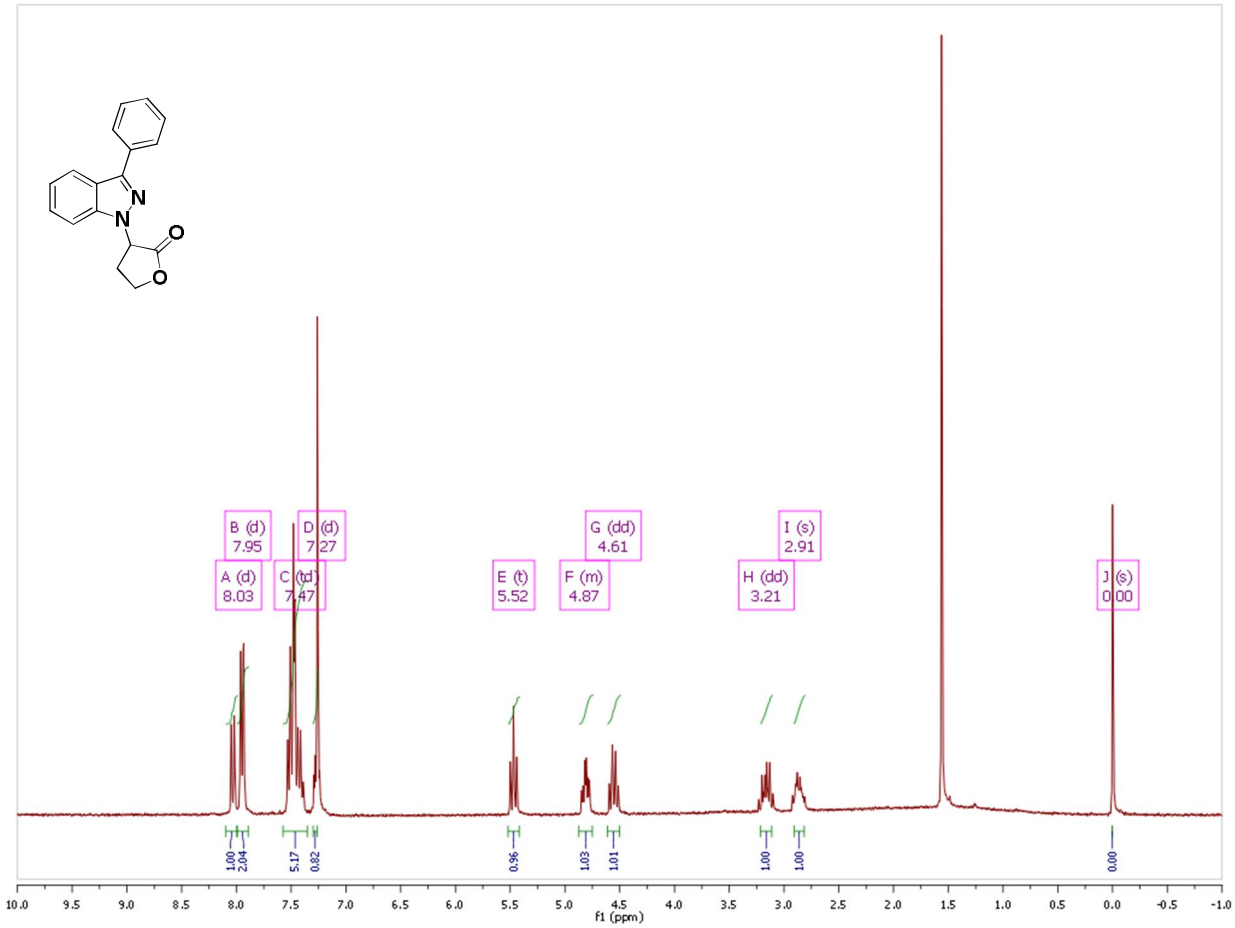
^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 49835873 (Table 4, entry 8)



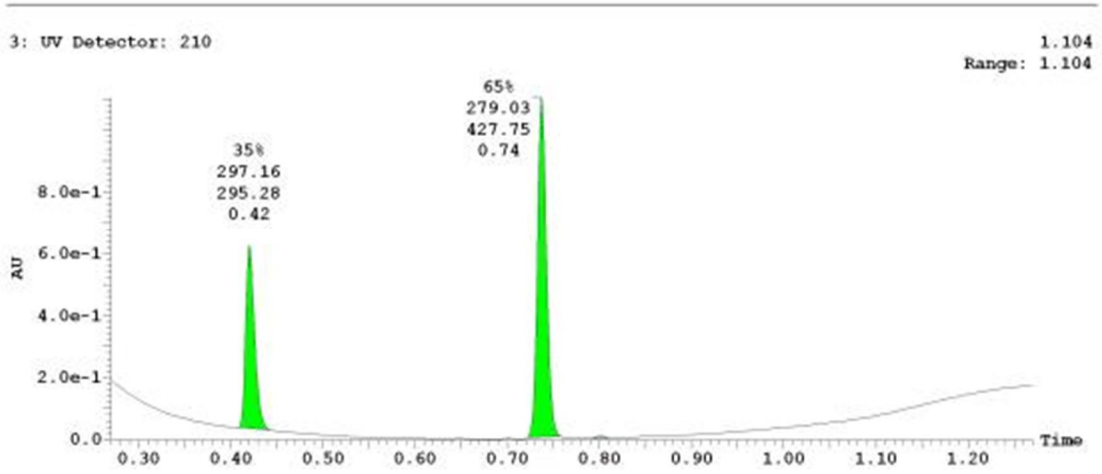
UPLC-MS of Analog CID 49835873



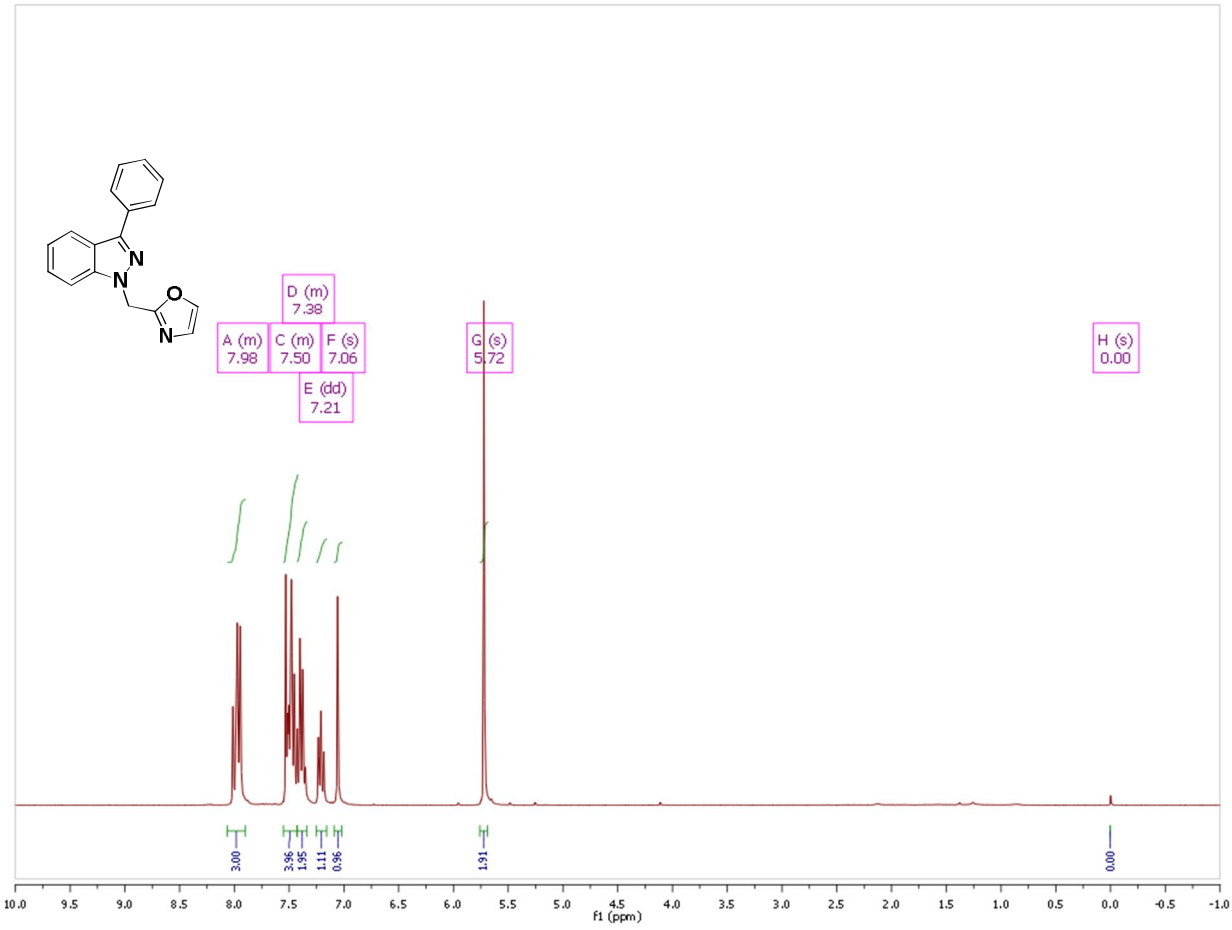
^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 49835834 (Table 4, entry 9)



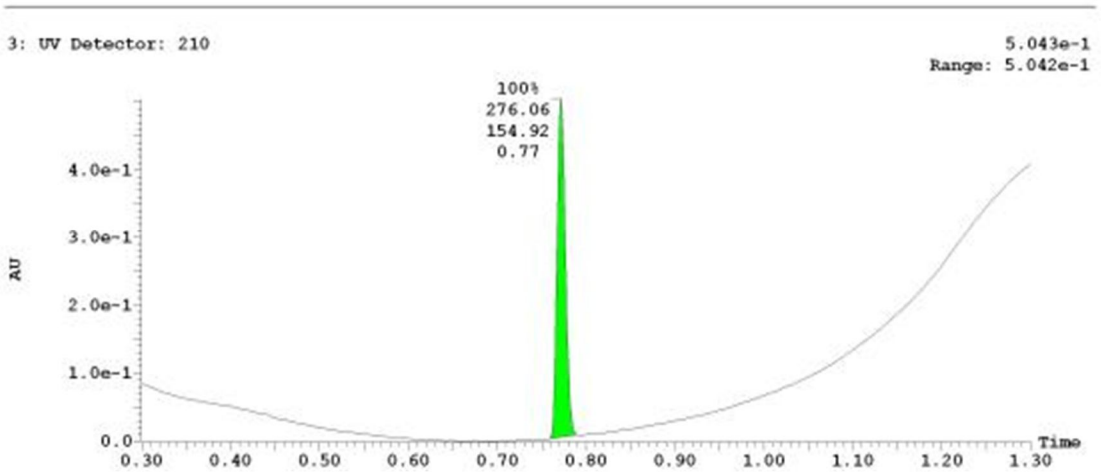
UPLC-MS of Analog CID 49835834



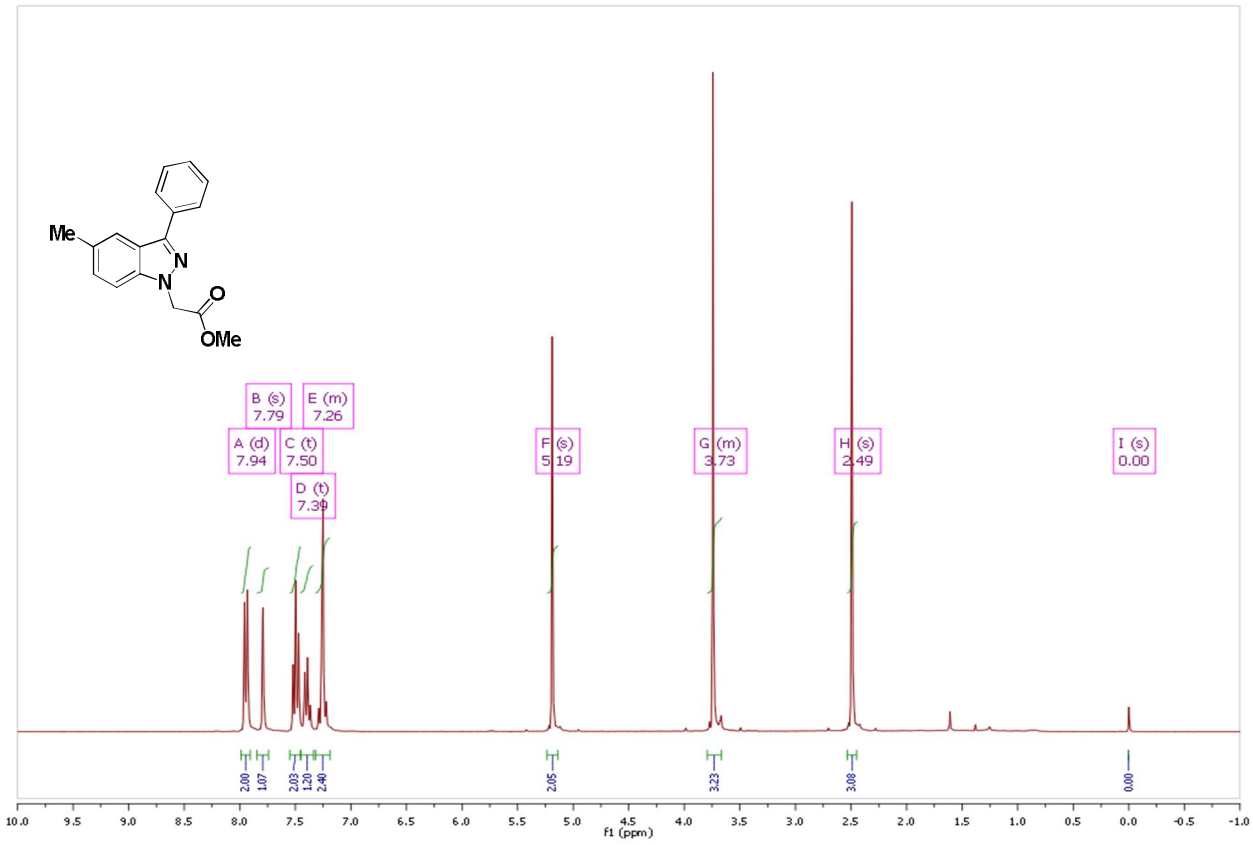
^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 49835836 (Table 4, entry 10)



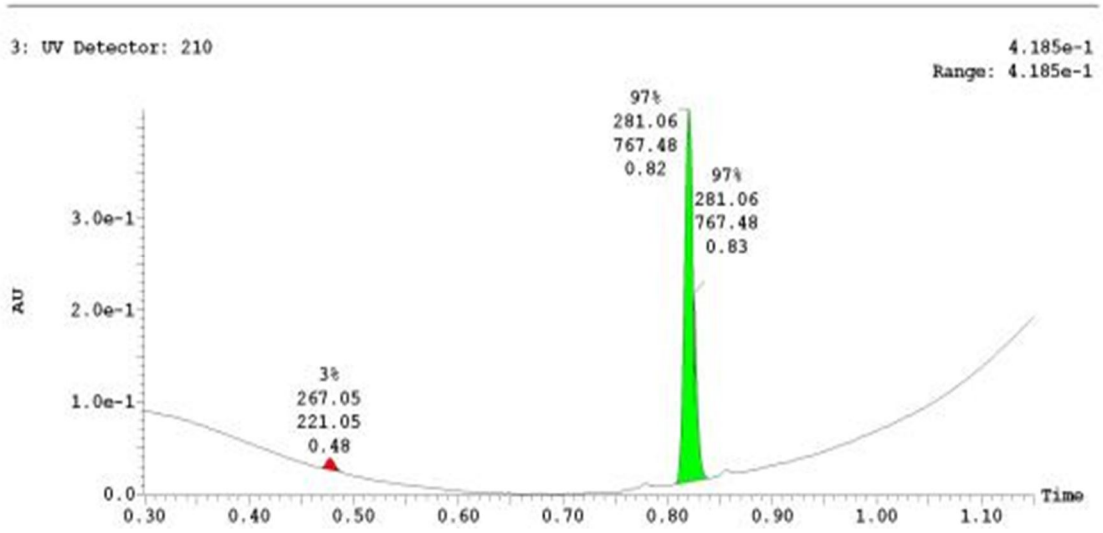
UPLC-MS of Analog CID 49835836



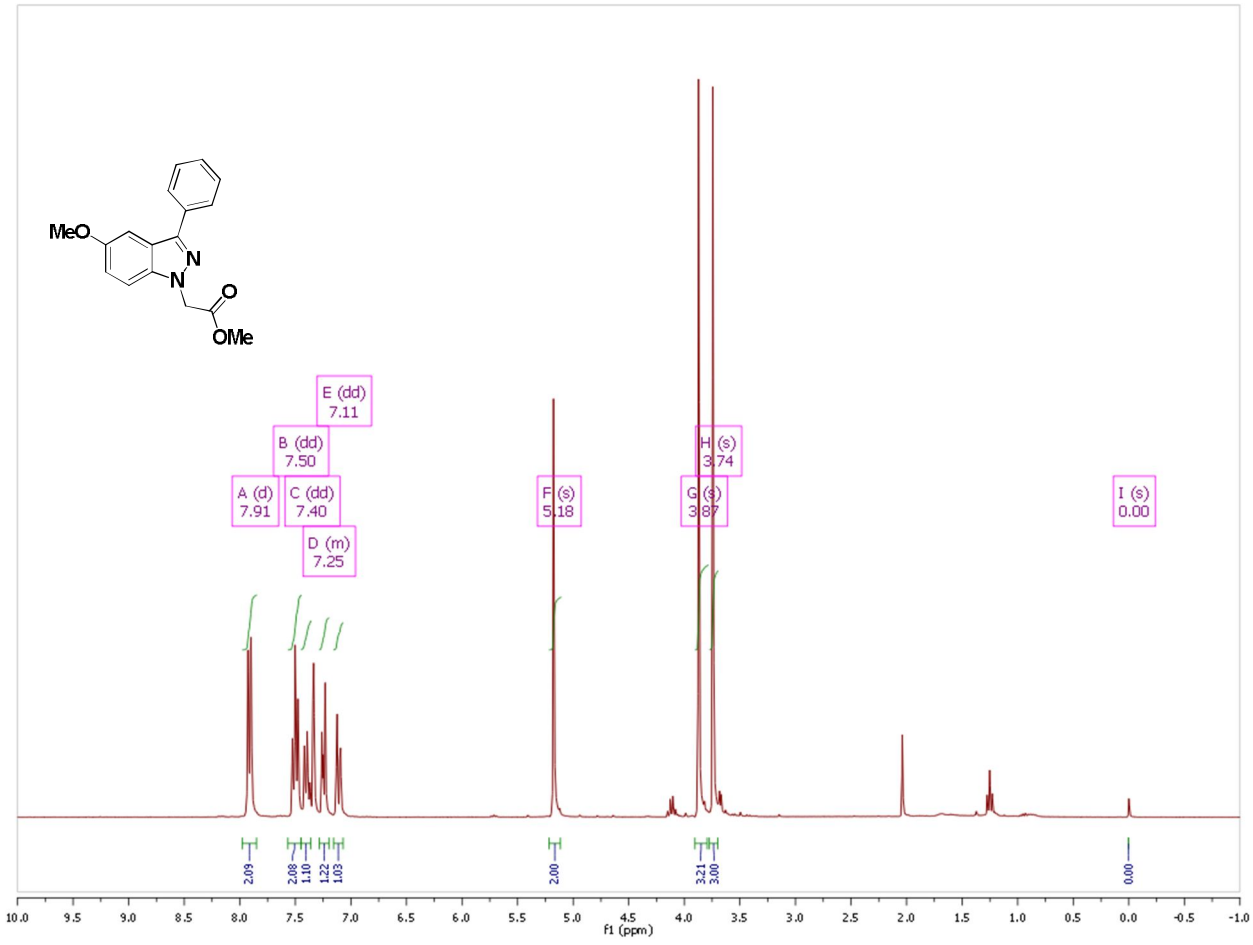
^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 49835874 (Table 5, entry 1)



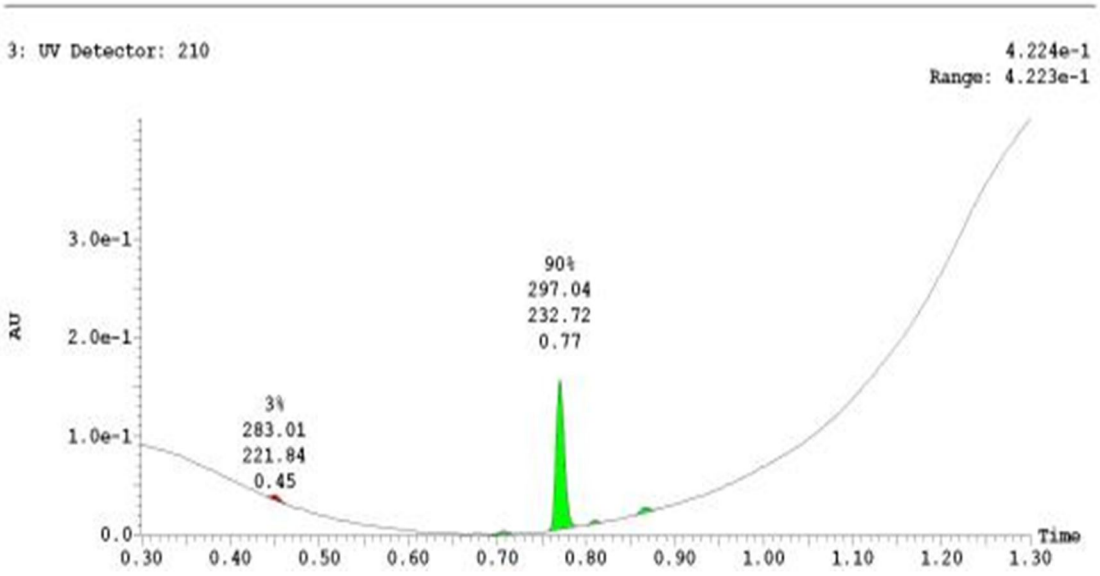
UPLC-MS of Analog CID 49835874



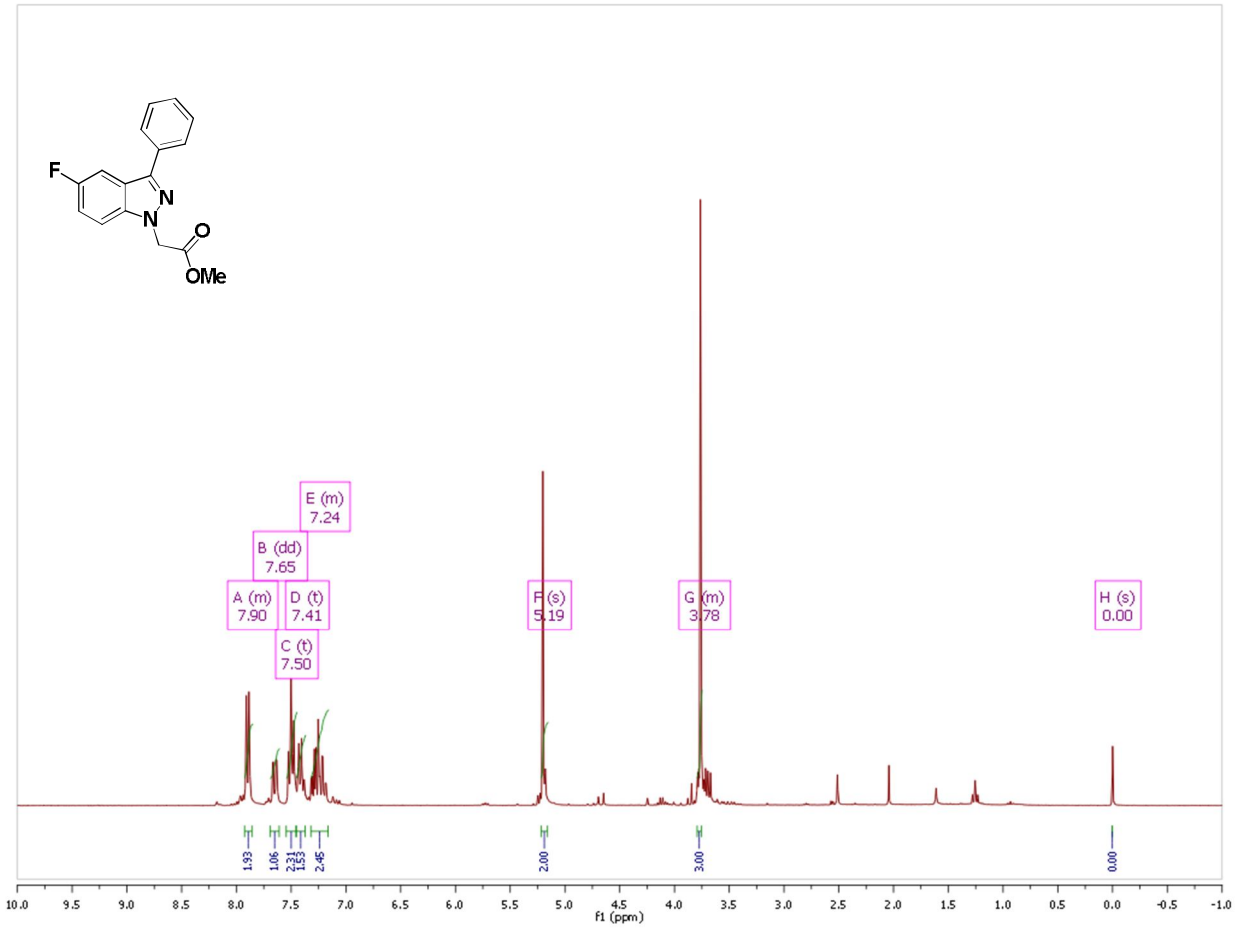
^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 49835837 (Table 5, entry 2)



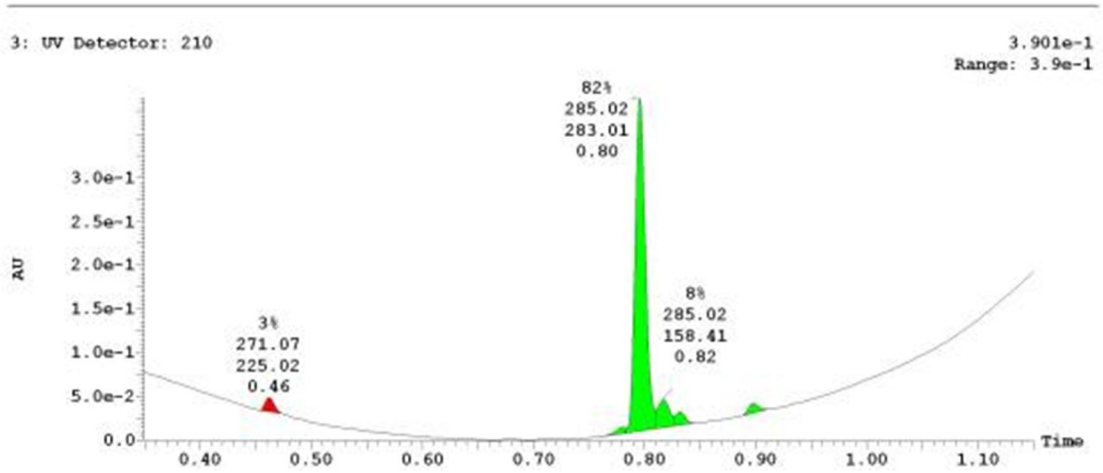
UPLC-MS of Analog CID 49835837



^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 49835889 (Table 5, entry 3)

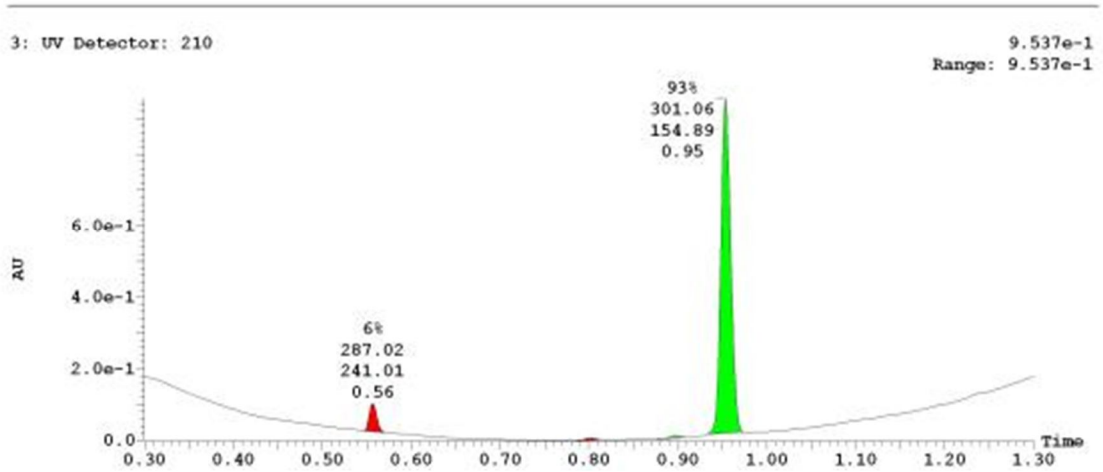


UPLC-MS of Analog CID 49835889



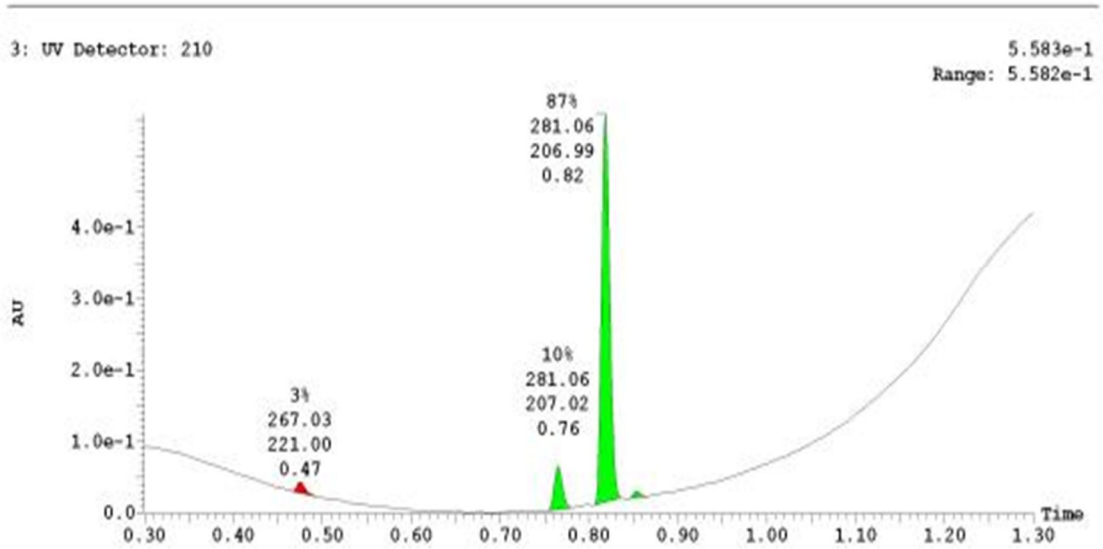
^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 9551137 (Table 5, entry 4)

UPLC-MS of Analog CID 9551137

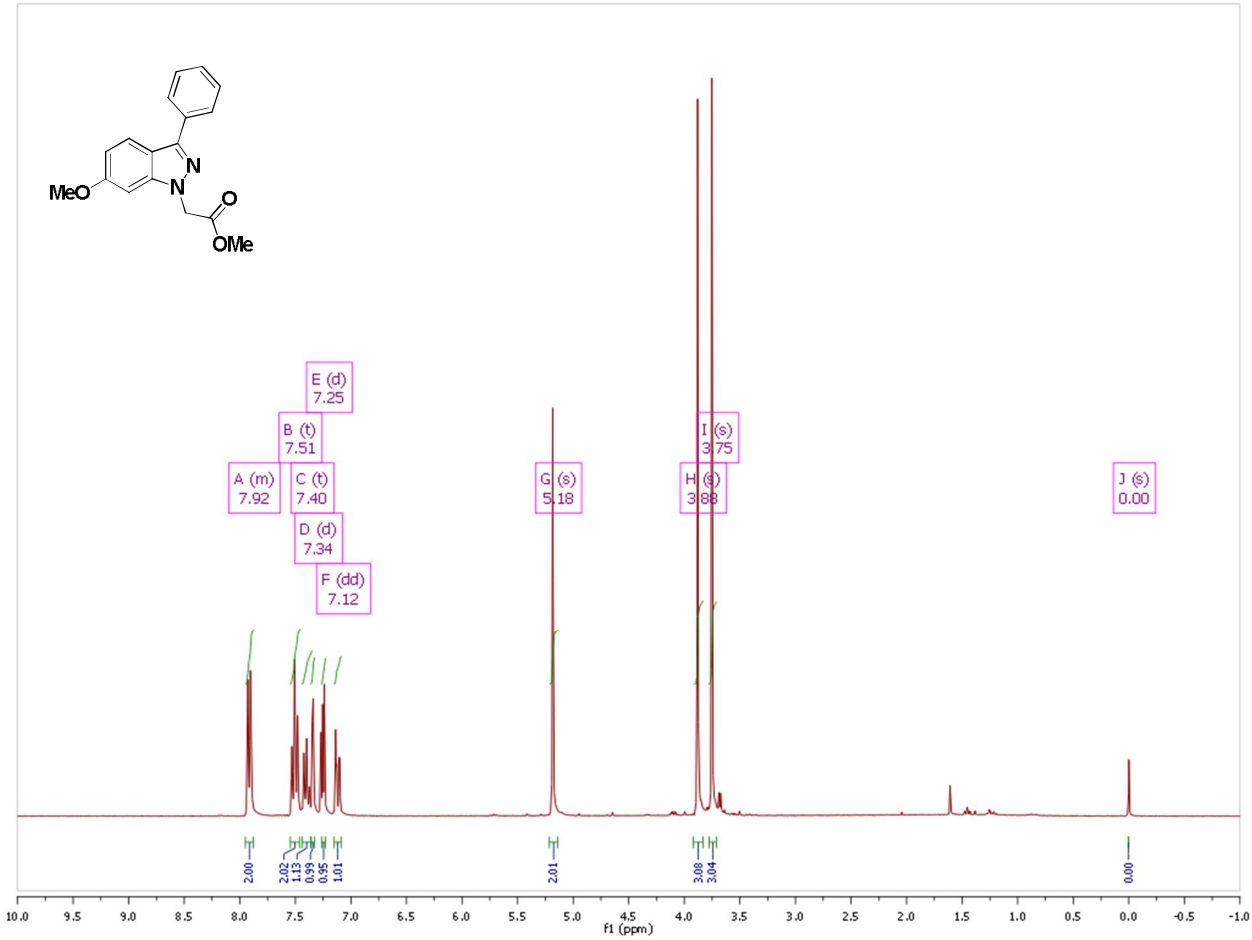


^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 49835872 (Table 5, entry 5)

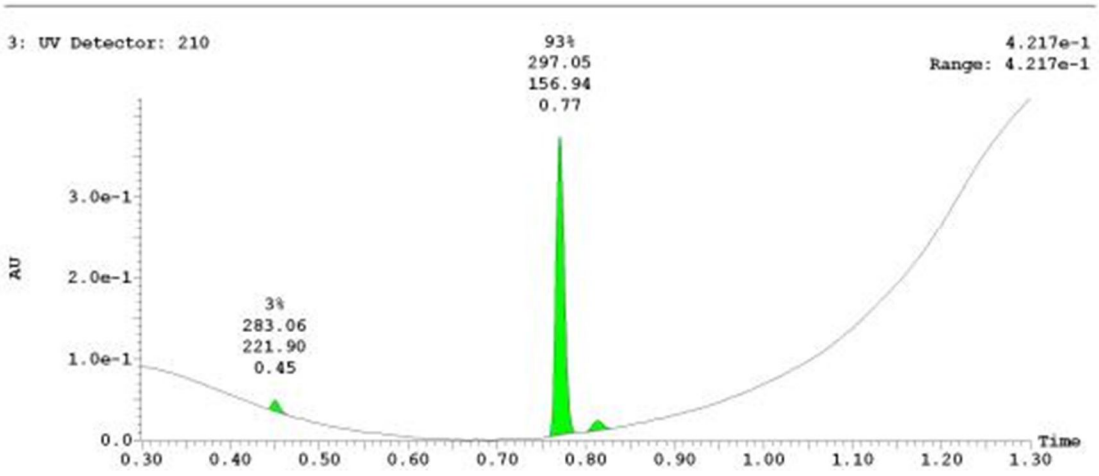
UPLC-MS of Analog CID 49835872



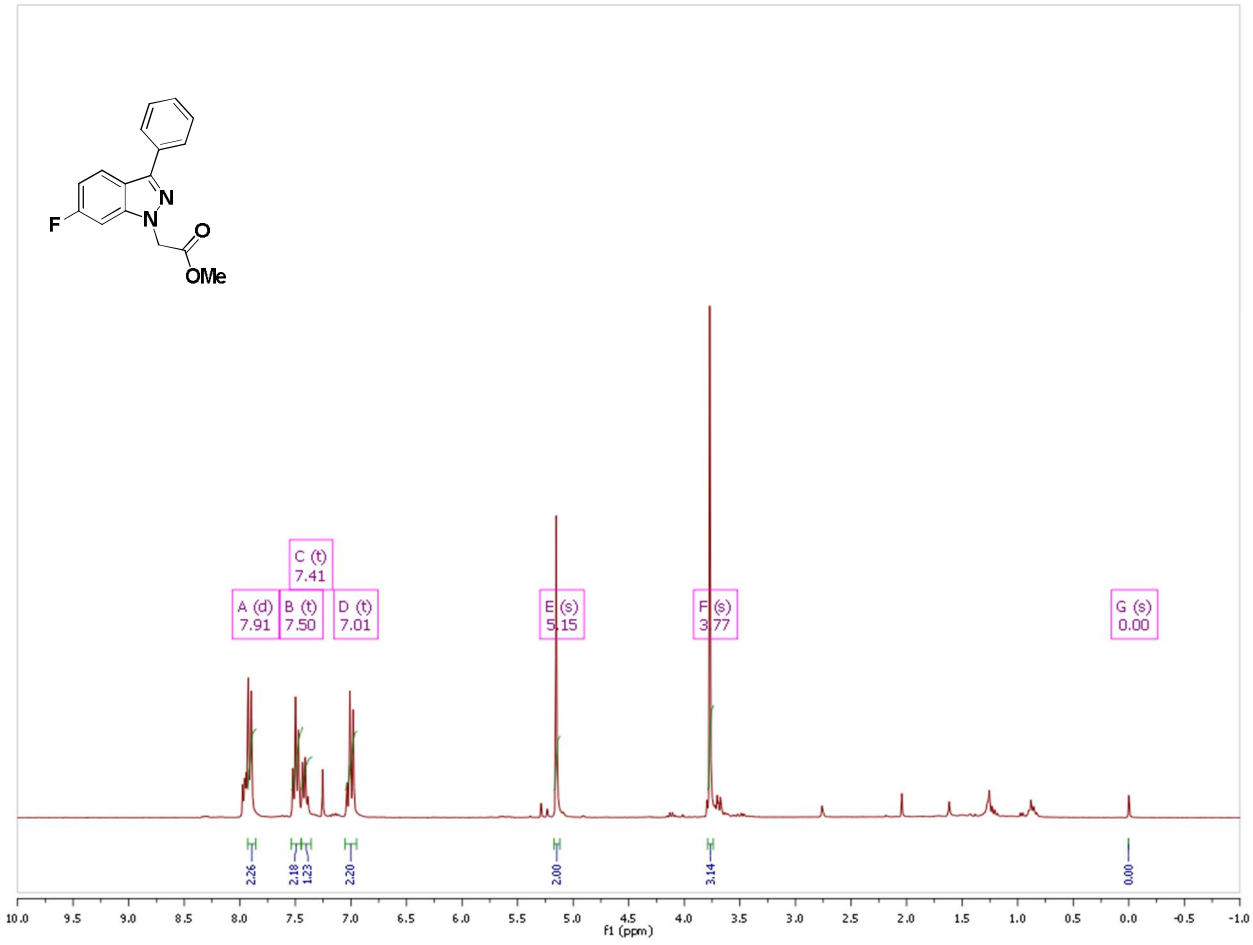
^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 49835812 (Table 5, entry 6)



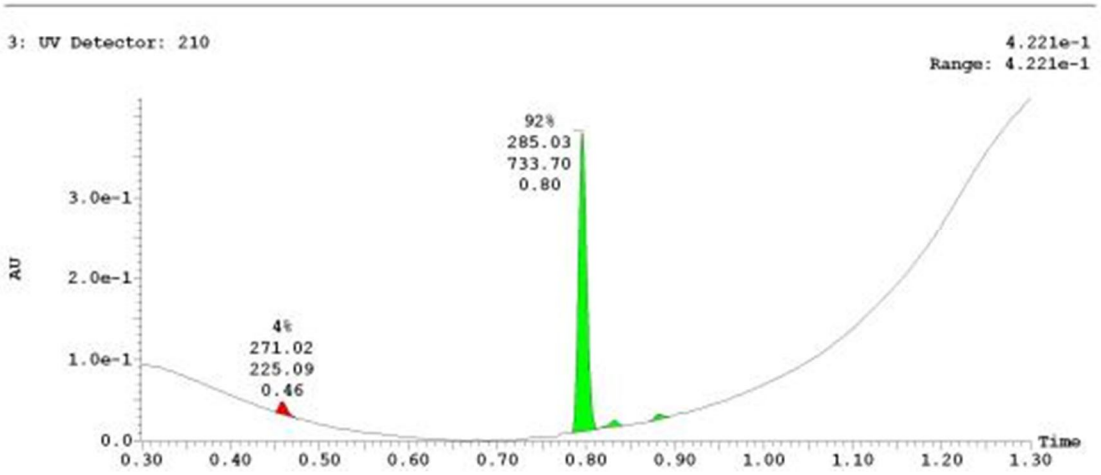
UPLC-MS of Analog CID 49835812



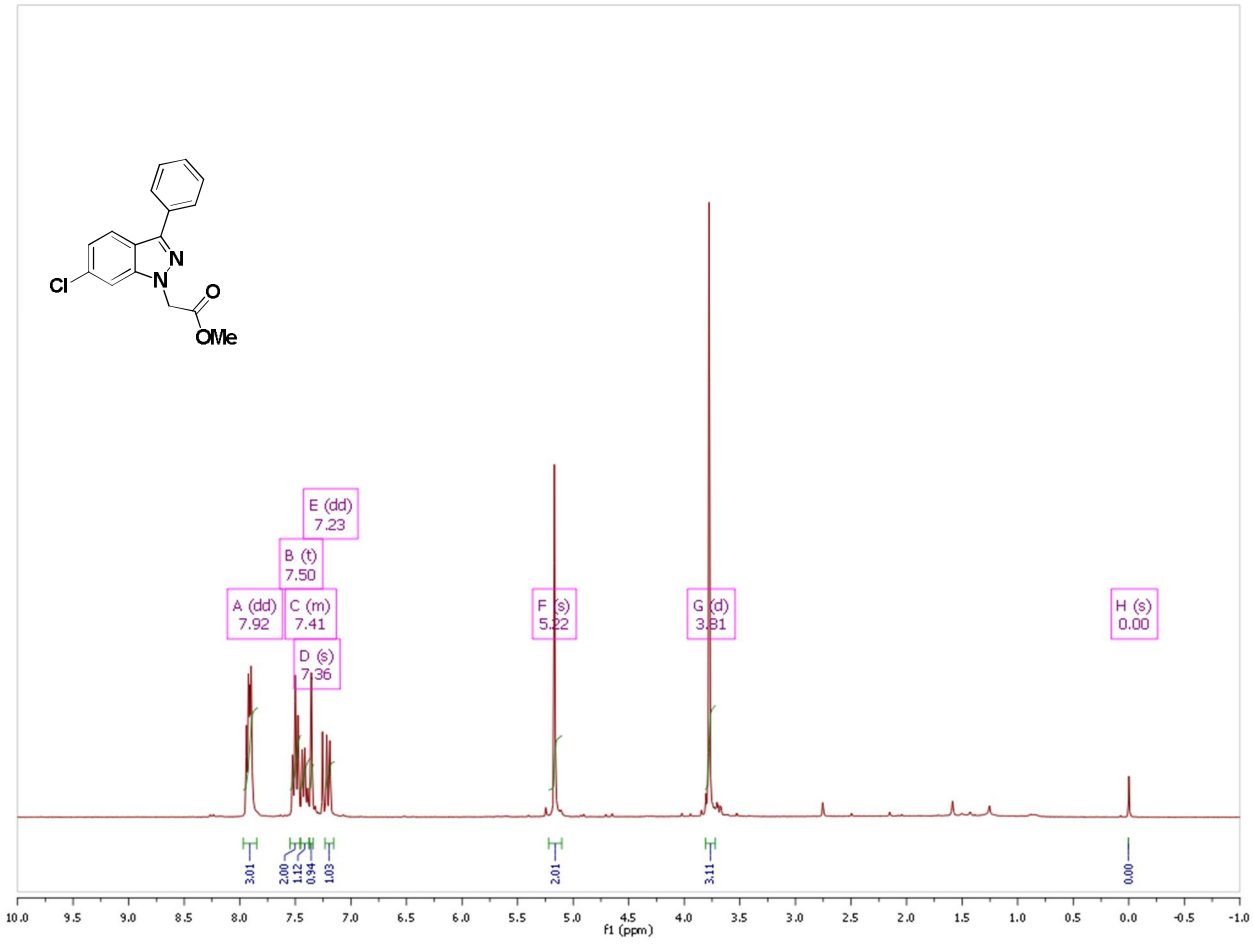
^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 49835815 (Table 5, entry 7)



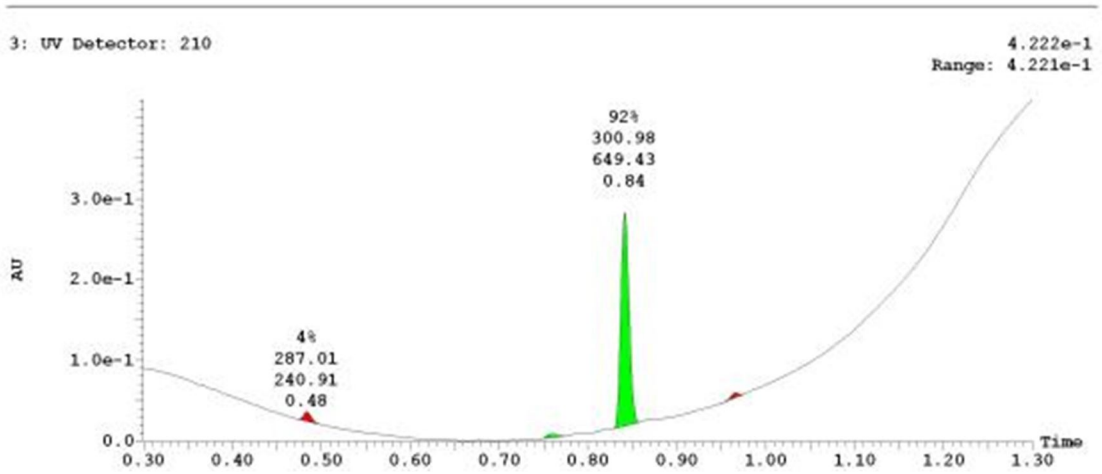
UPLC-MS of Analog CID 49835815



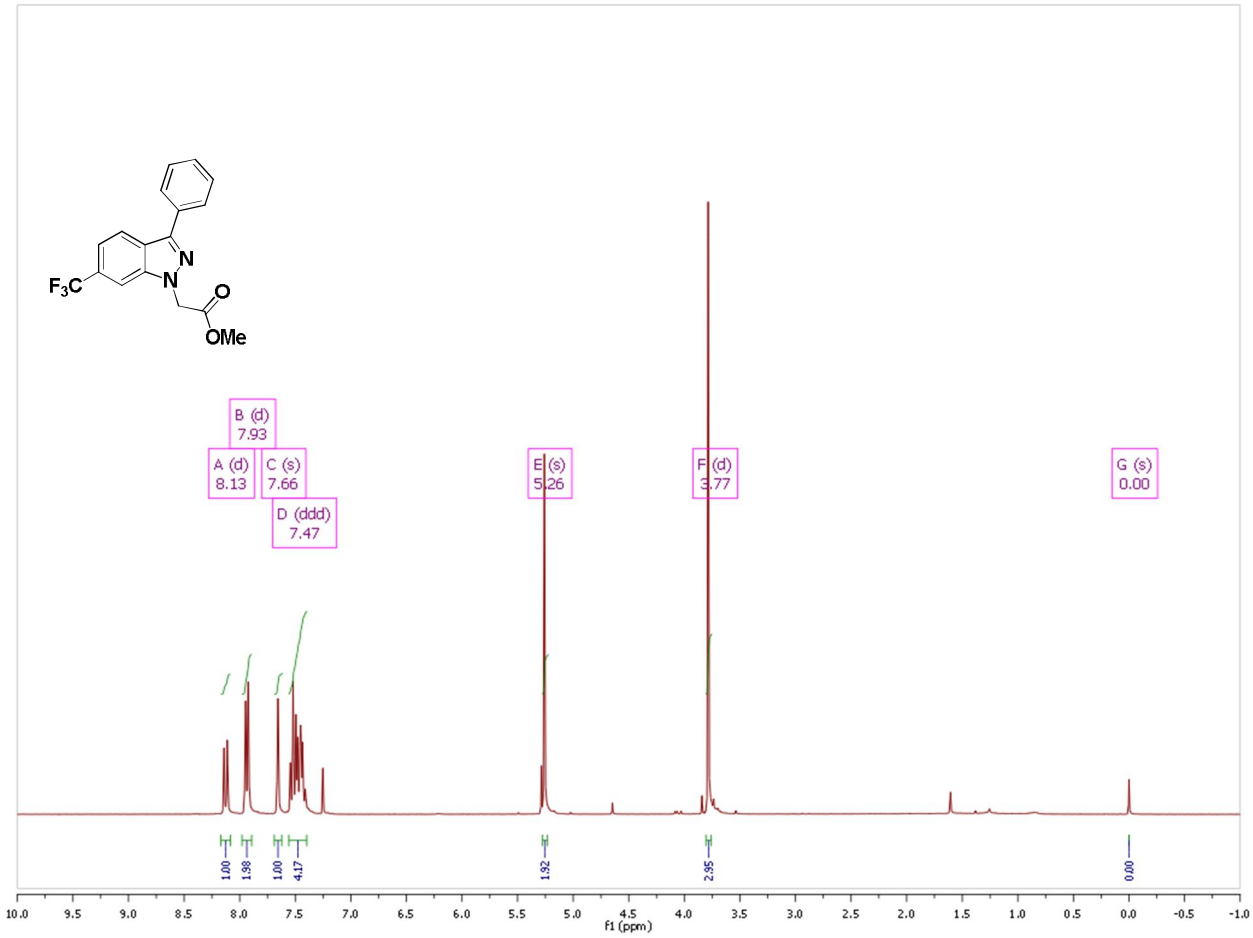
^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 49835818 (Table 5, entry 8)



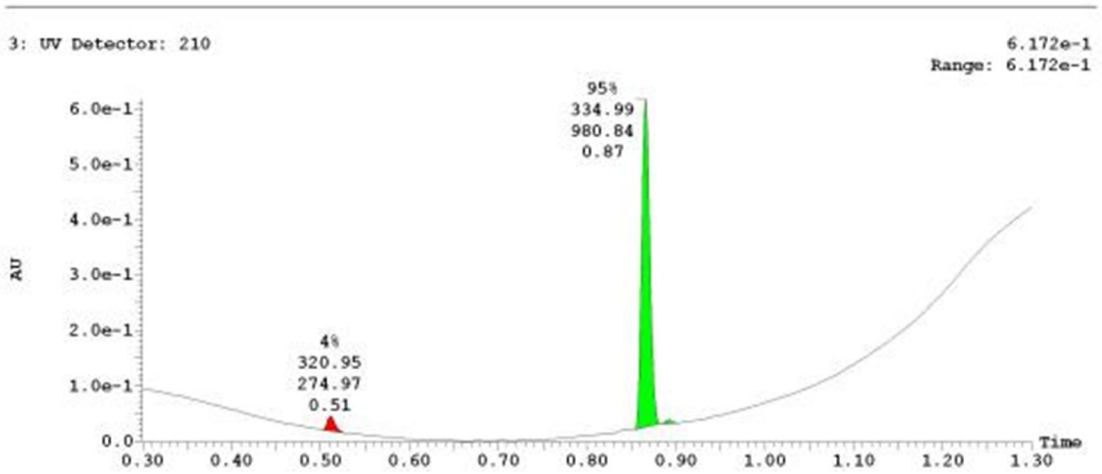
UPLC-MS of Analog CID 49835818



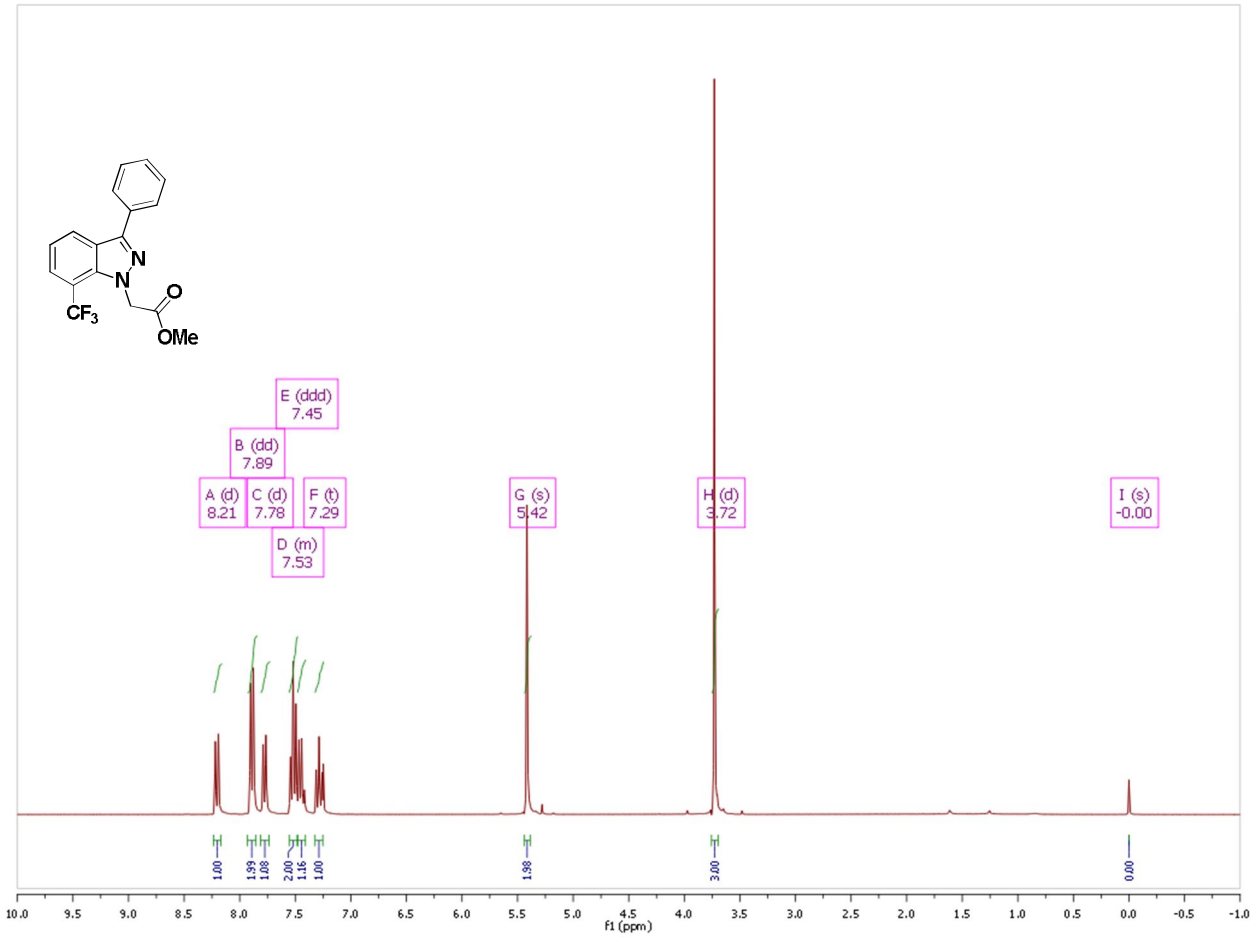
^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 49835849 (Table 5, entry 9)



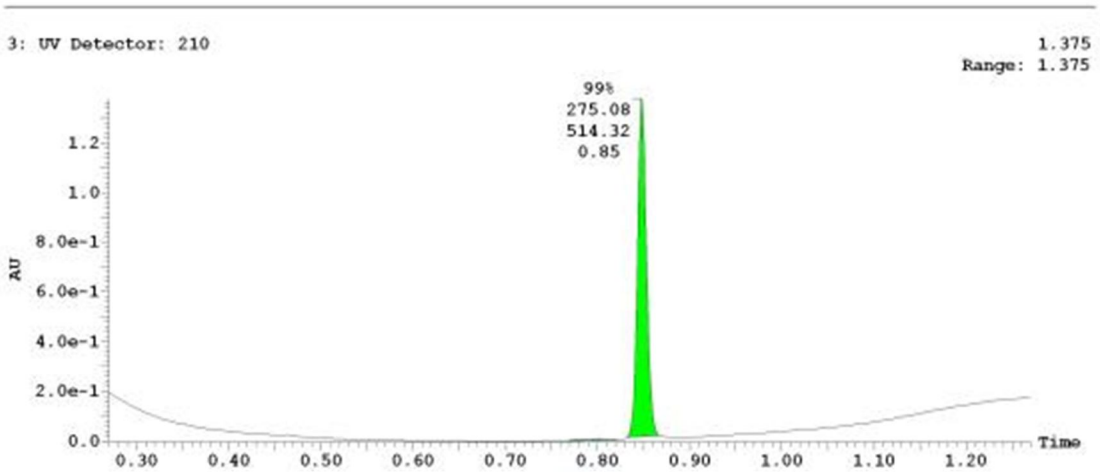
UPLC-MS of Analog CID 49835849



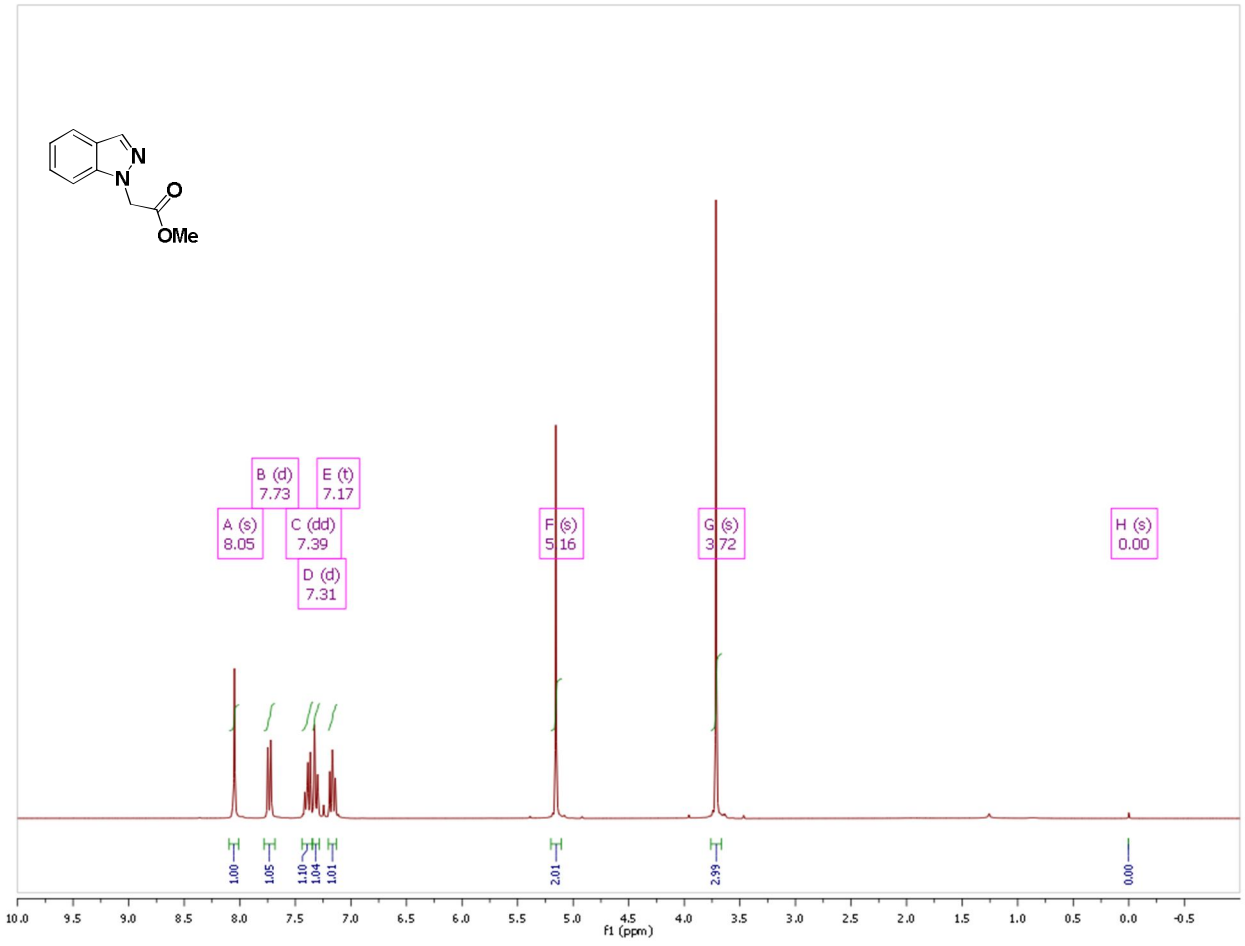
^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 49835842 (Table 5, entry 10)



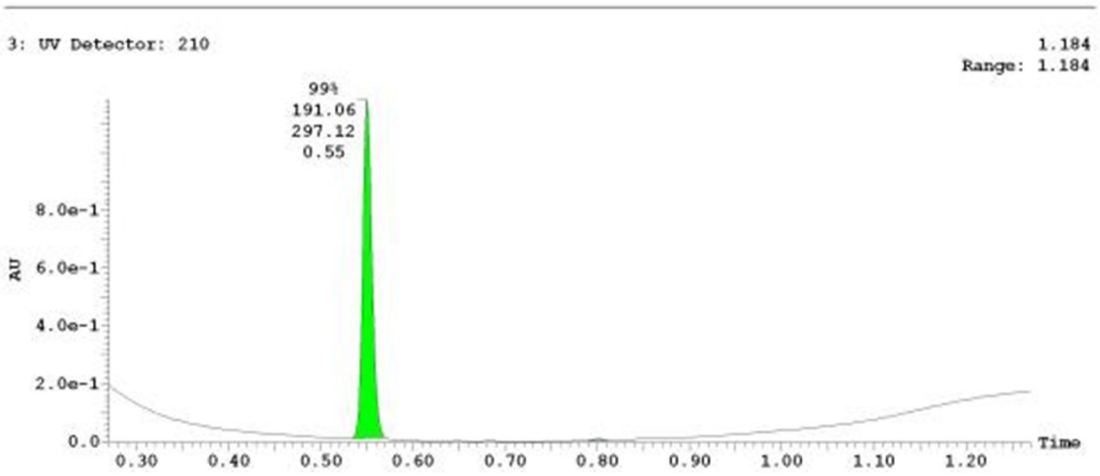
UPLC-MS of Analog CID 49835842



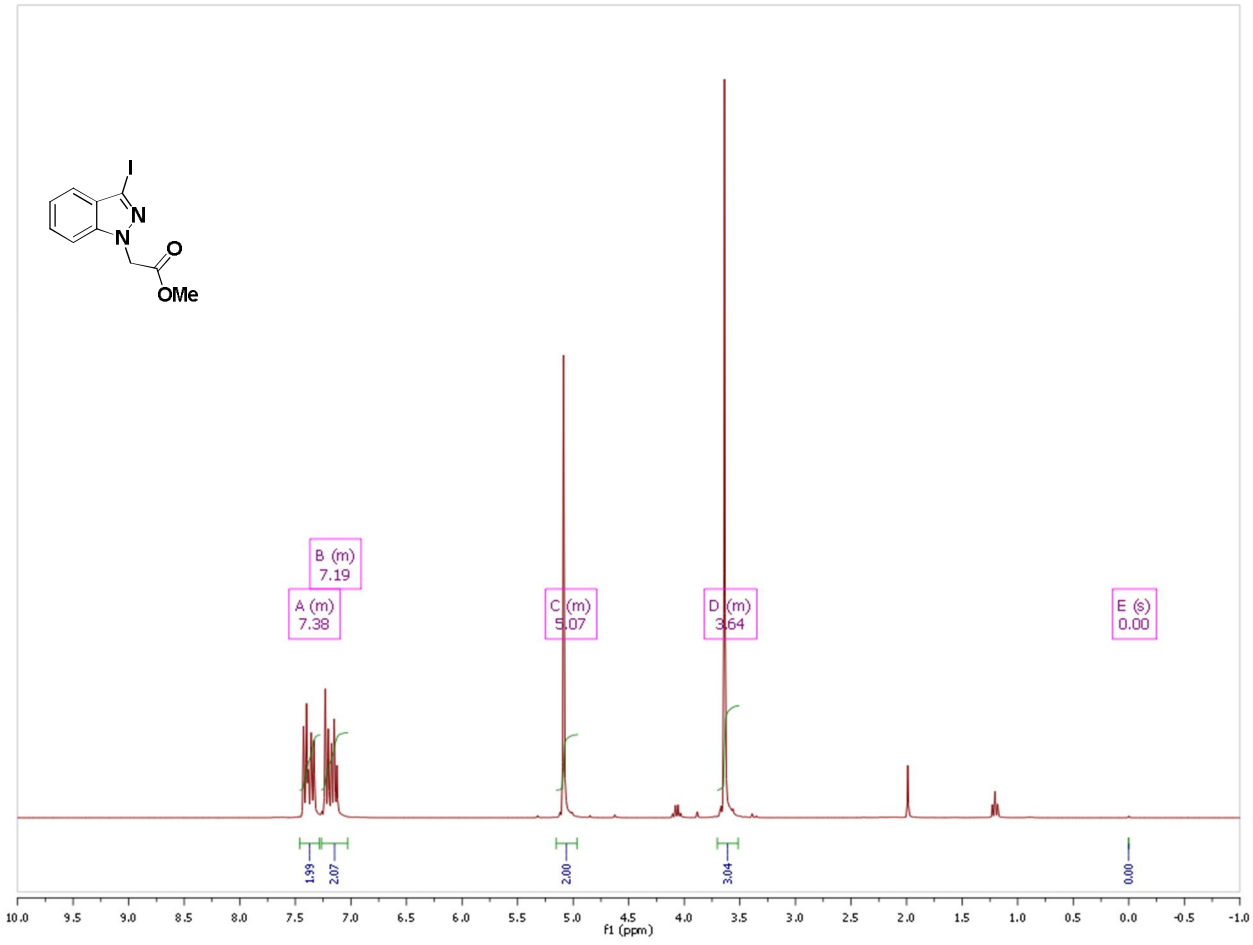
^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 49835852 (Table 6, entry 1)



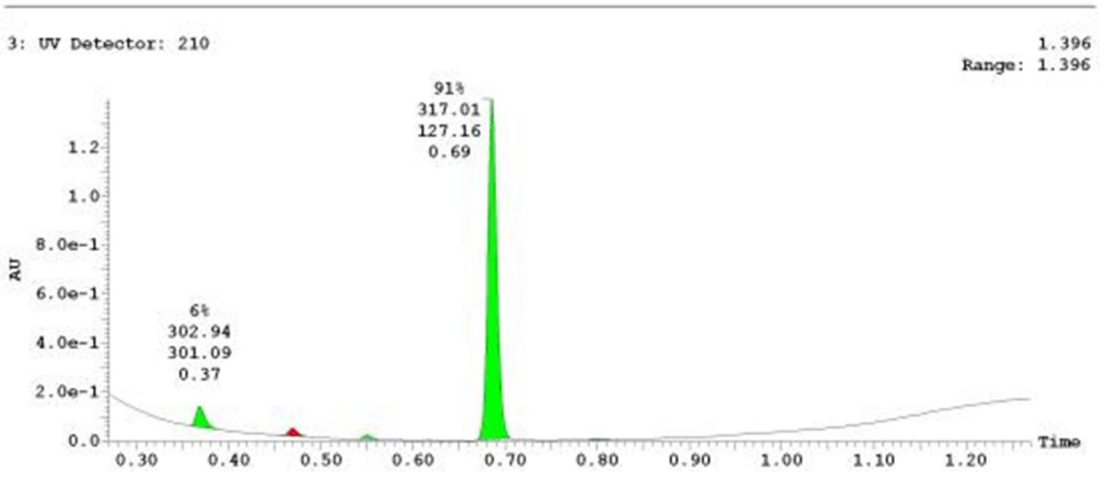
UPLC-MS of Analog CID 49835852



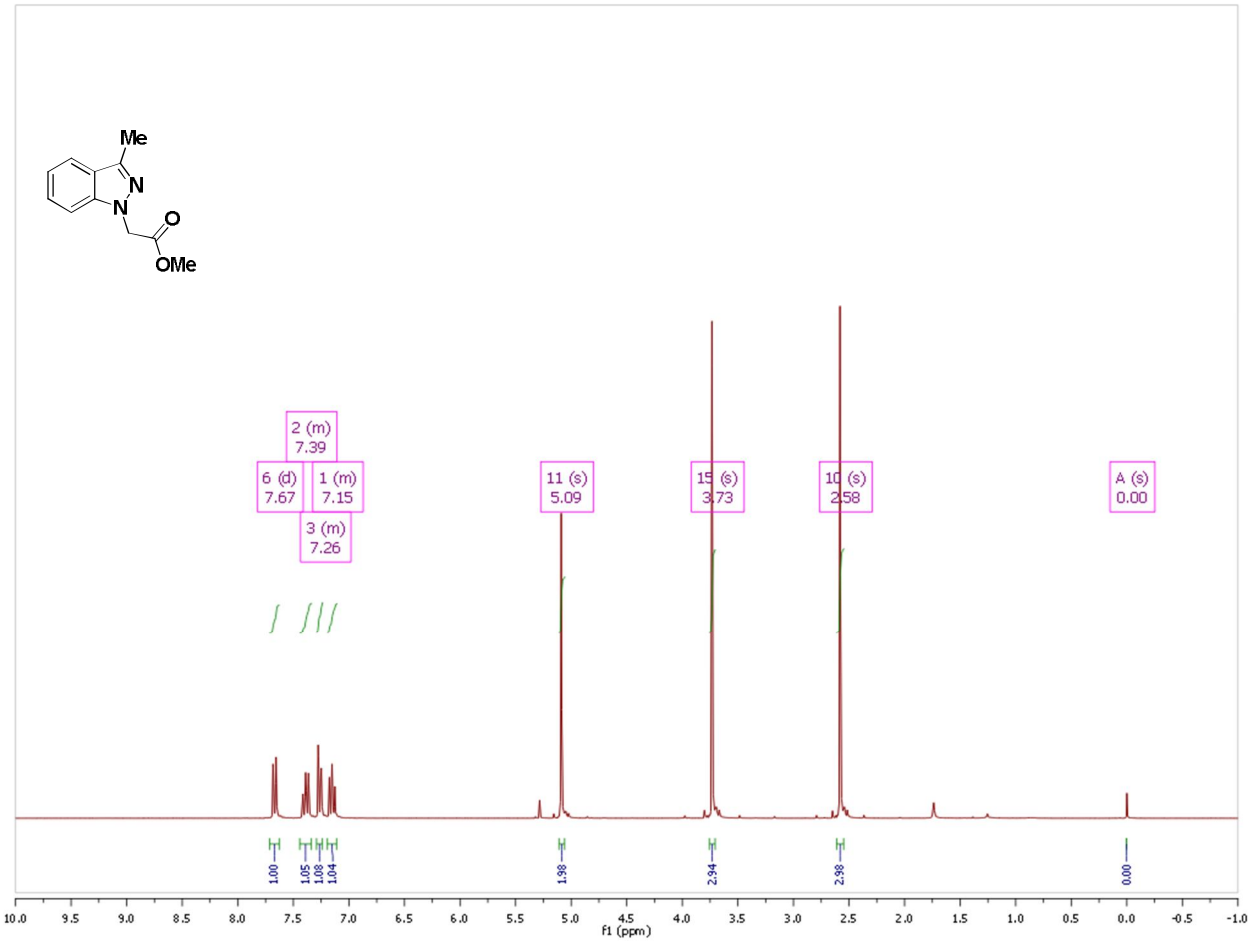
^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 49835832 (Table 6, entry 2)



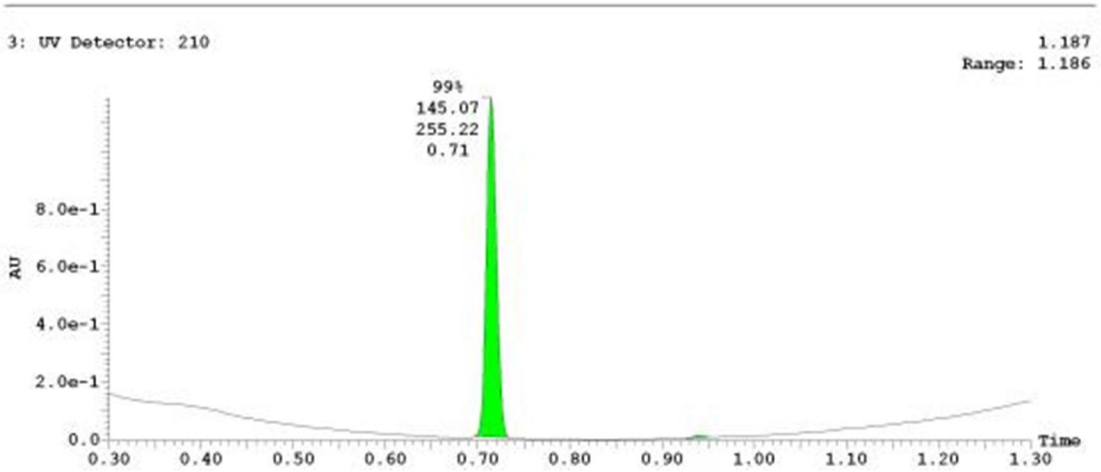
UPLC-MS of Analog CID 49835832



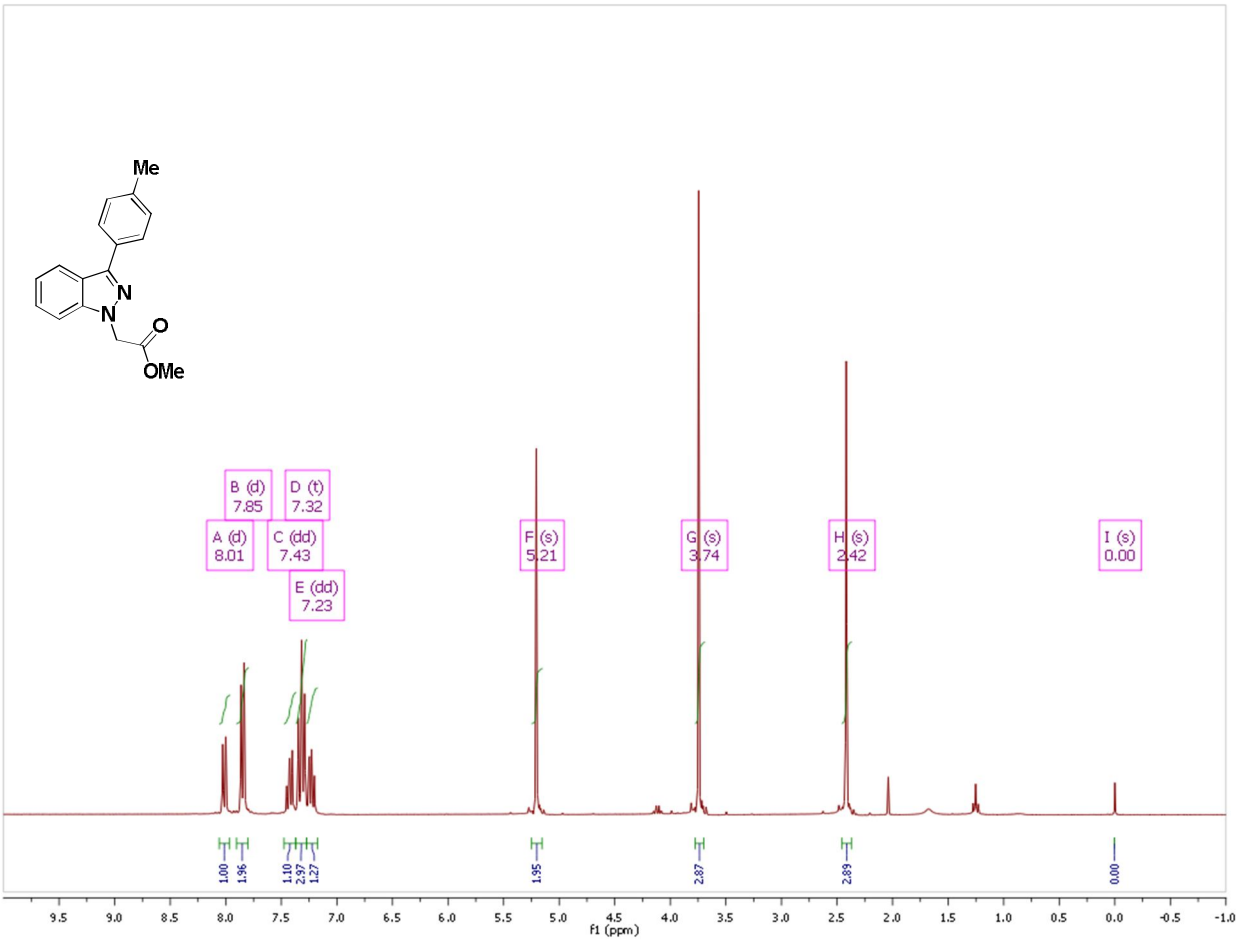
^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 46856254 (Table 6, entry 3)



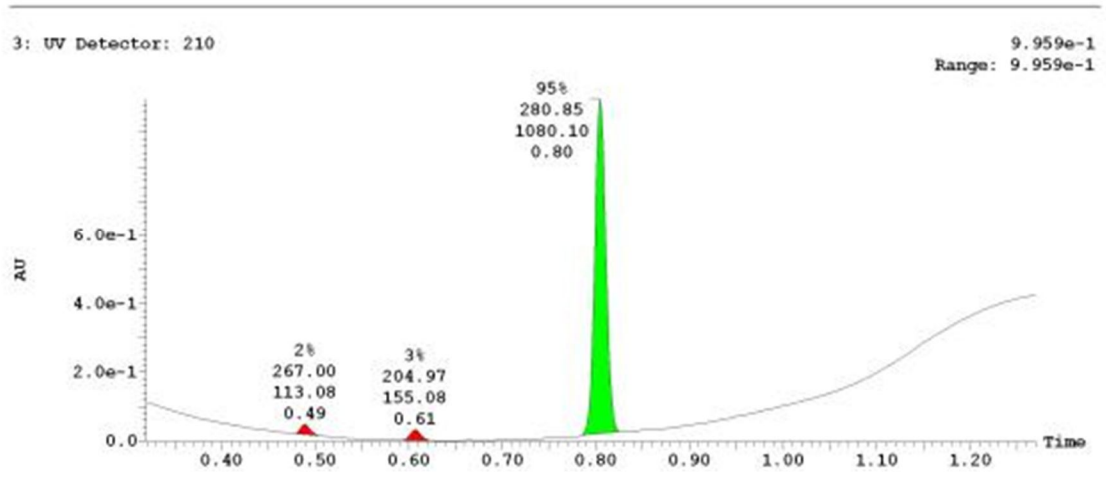
UPLC-MS of Analog CID 46856254



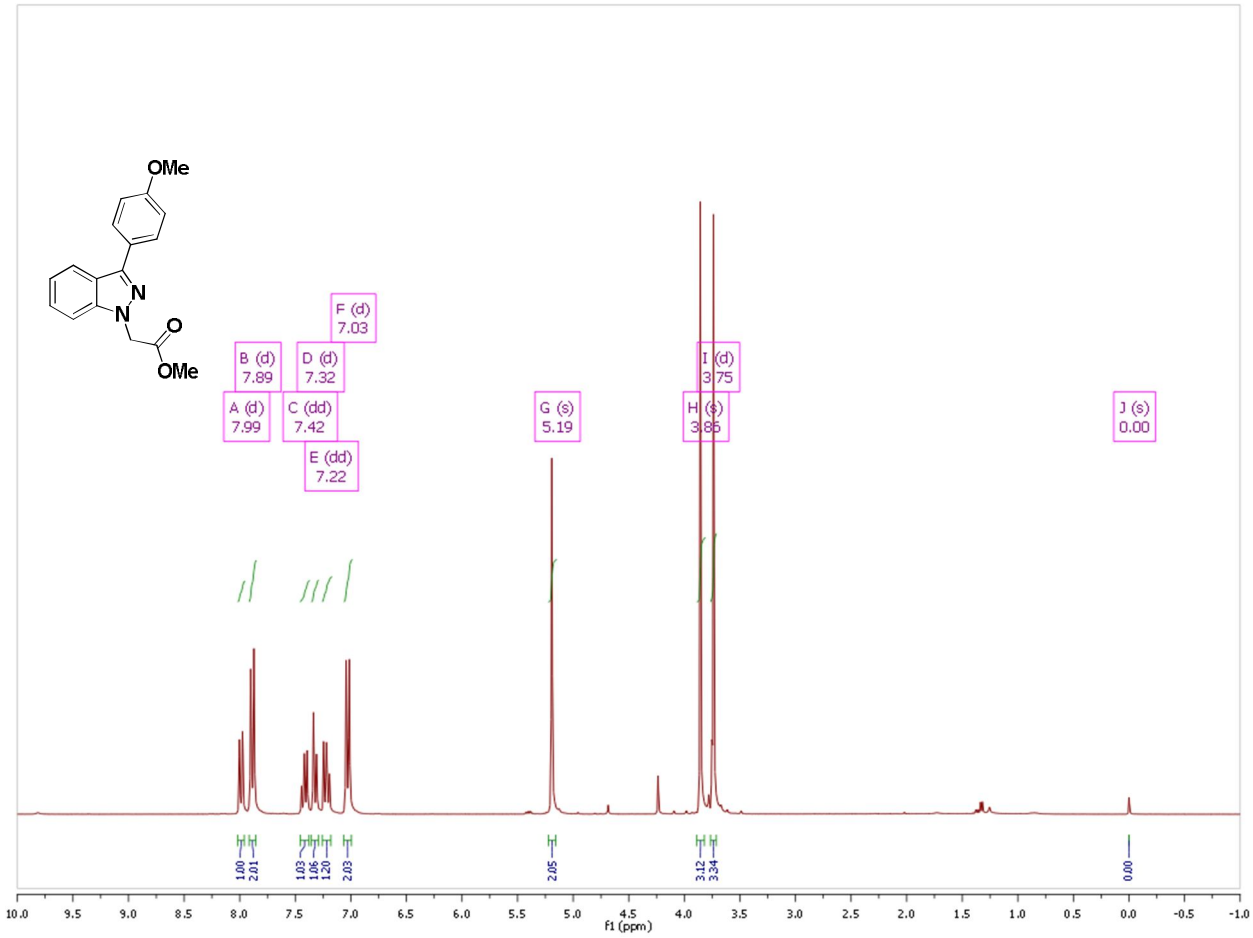
^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 46856255 (Table 7, entry 1)



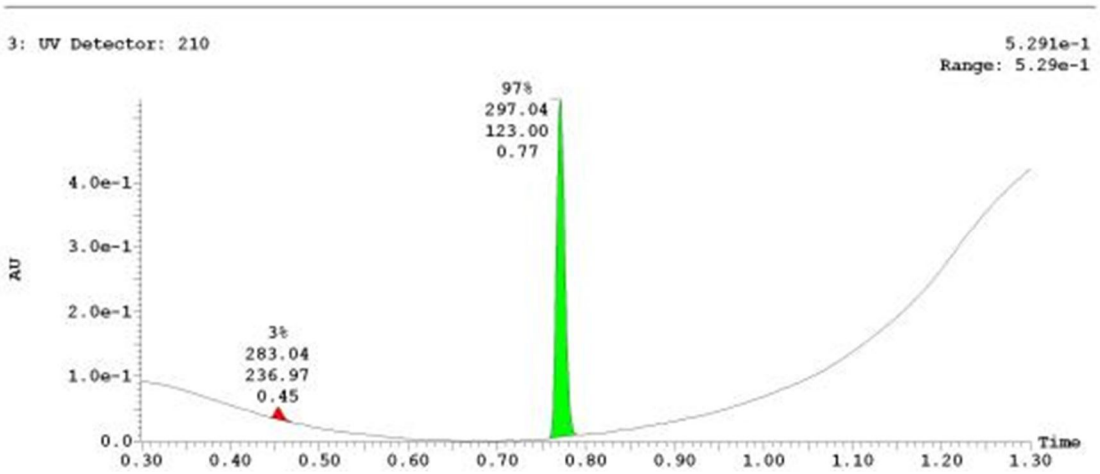
UPLC-MS of Analog CID 46856255



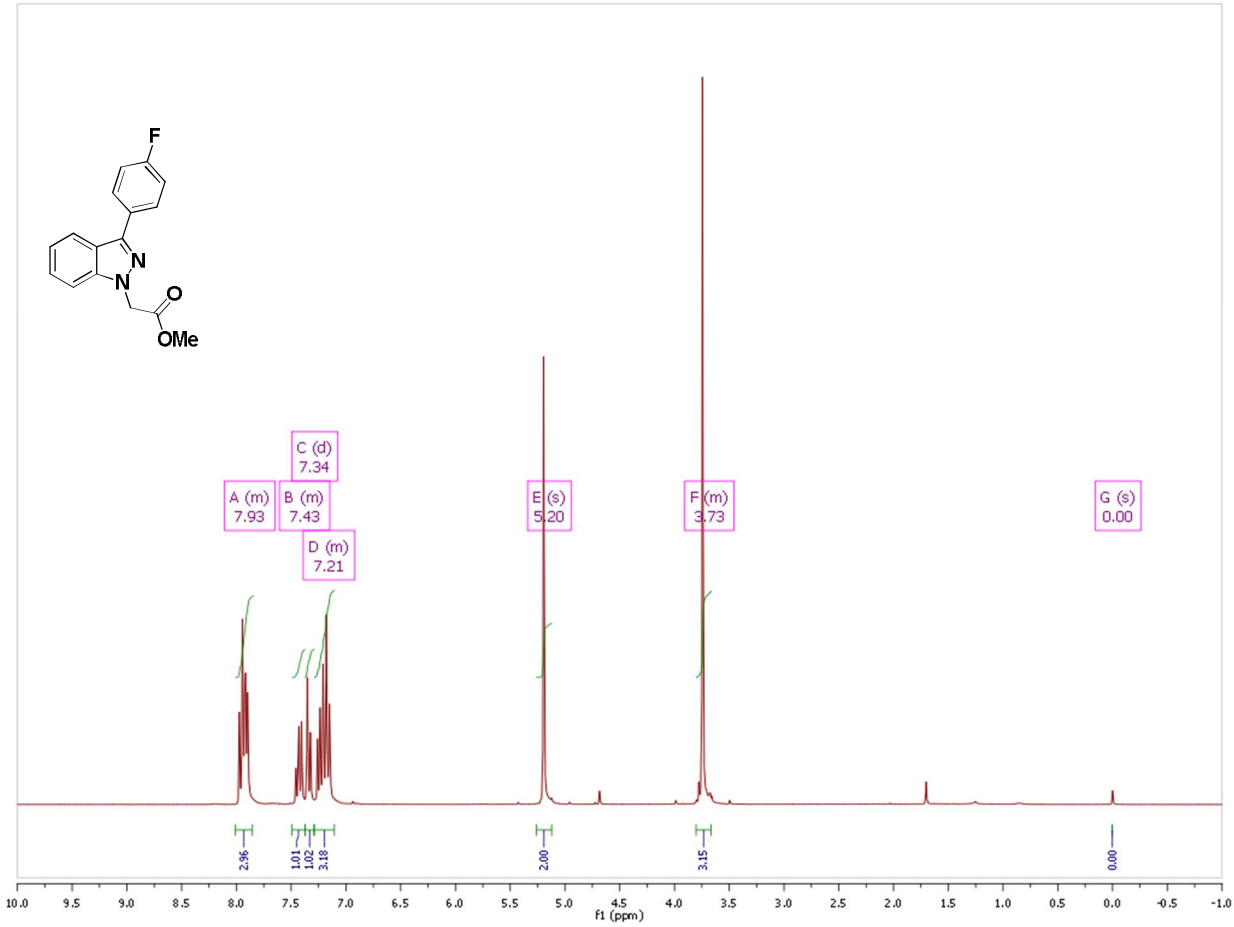
^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 49835864 (Table 7, entry 2)



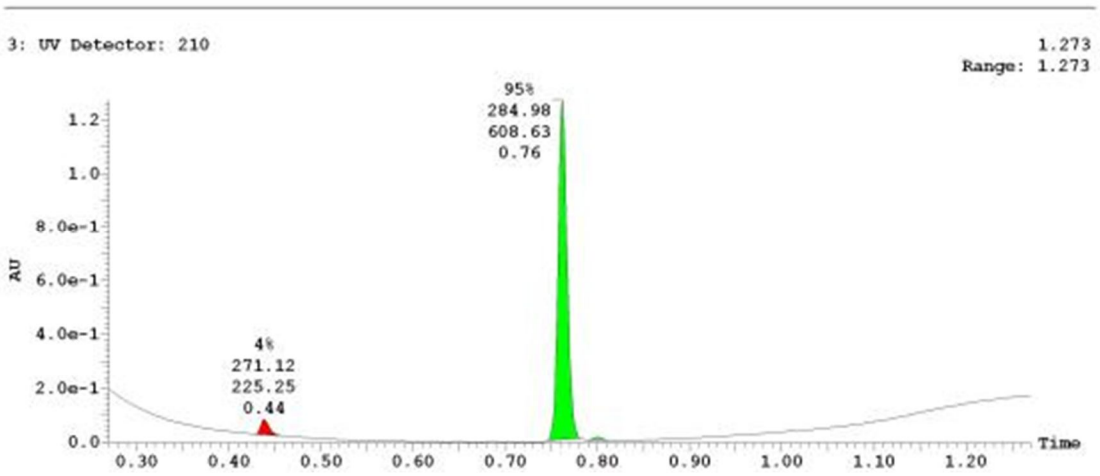
UPLC-MS of Analog CID 49835864



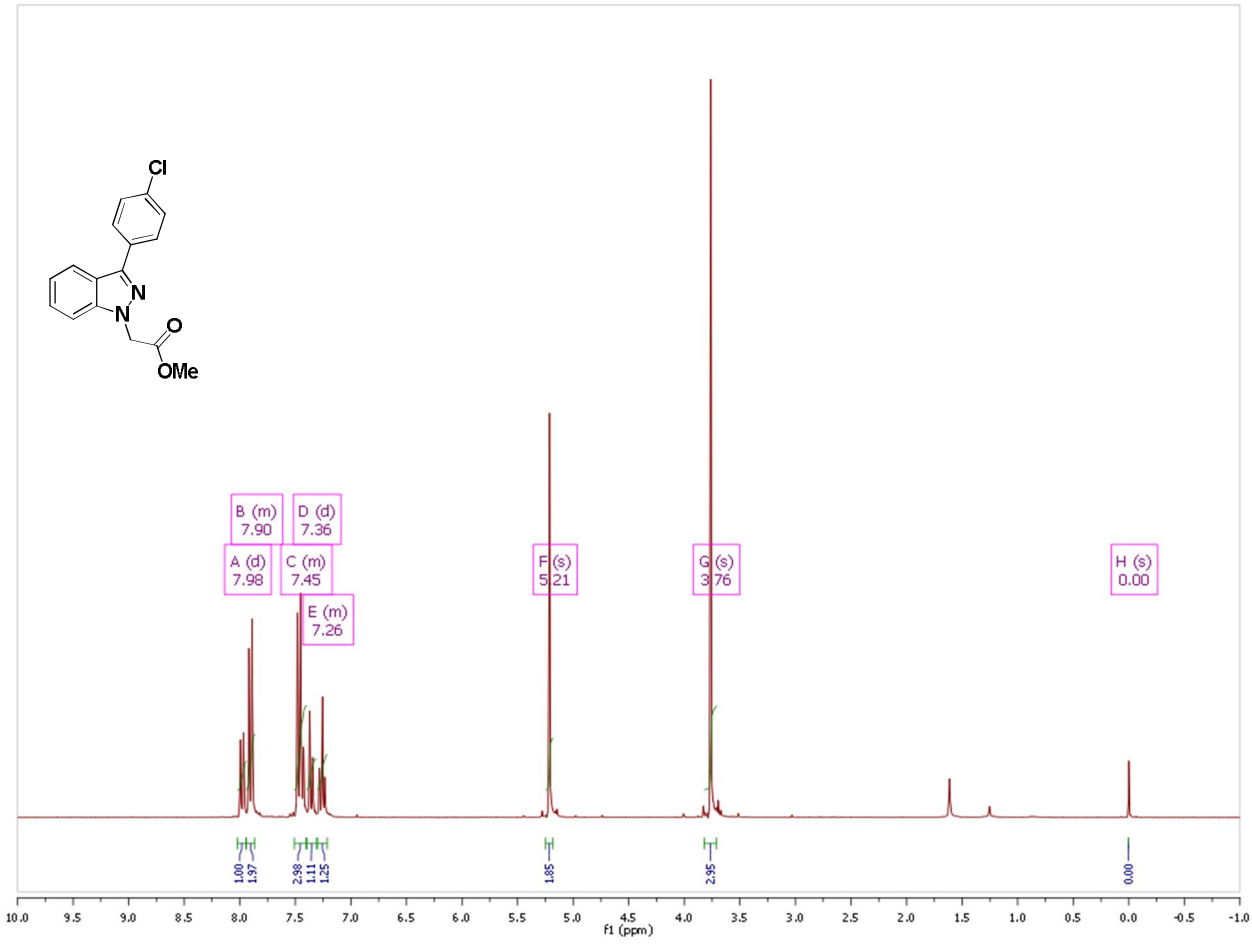
^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 49835876 (Table 7, entry 3)



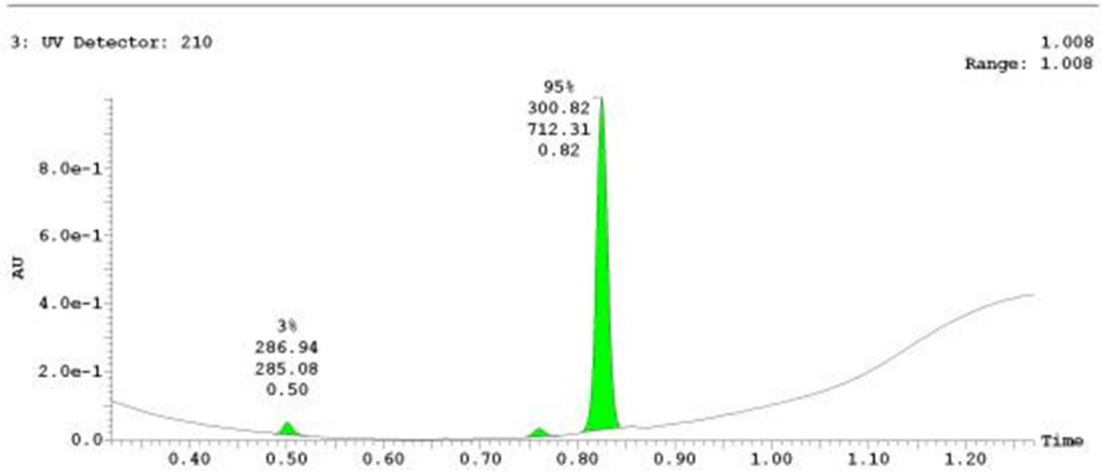
UPLC-MS of Analog CID 49835876



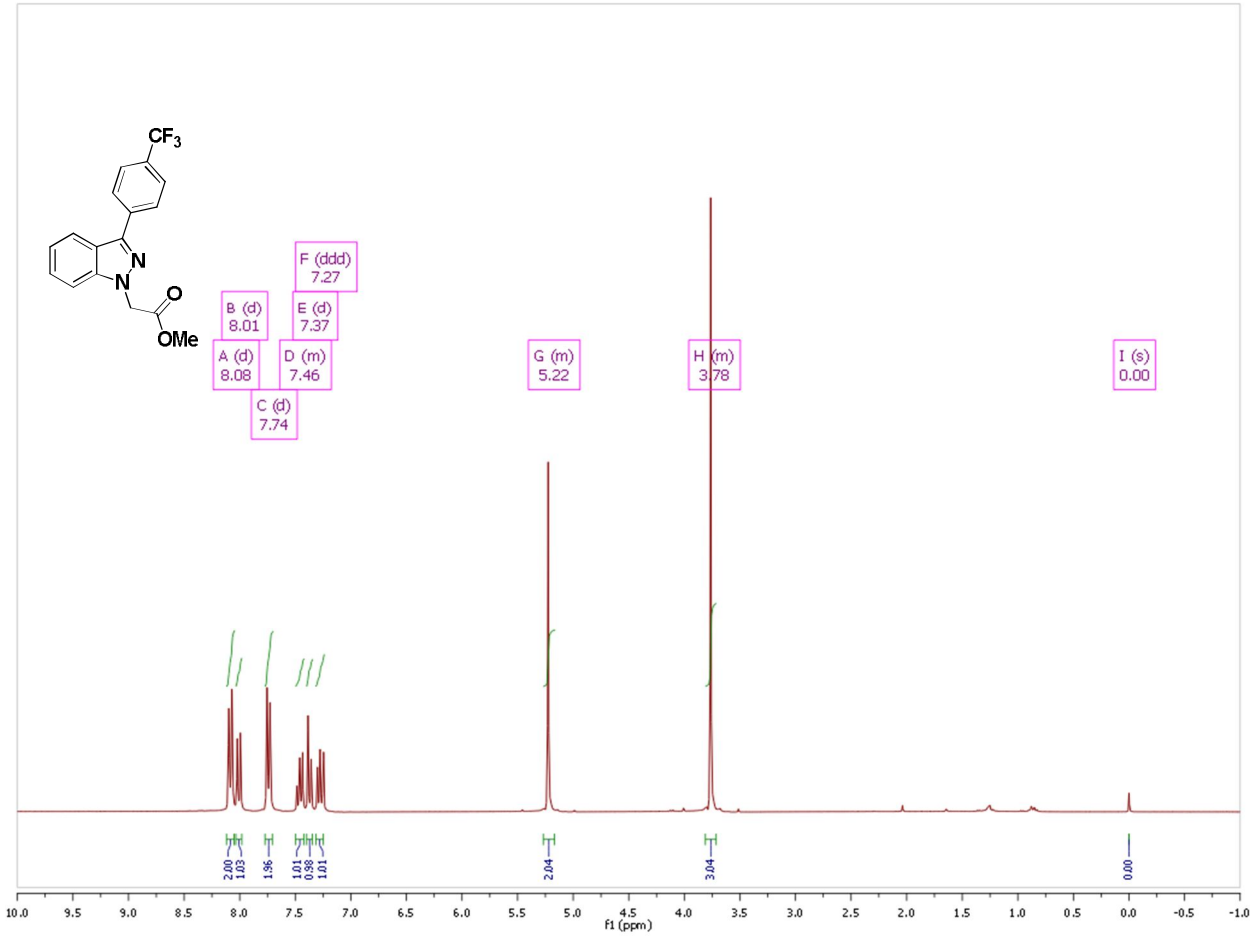
^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 46856253 (Table 7, entry 4)



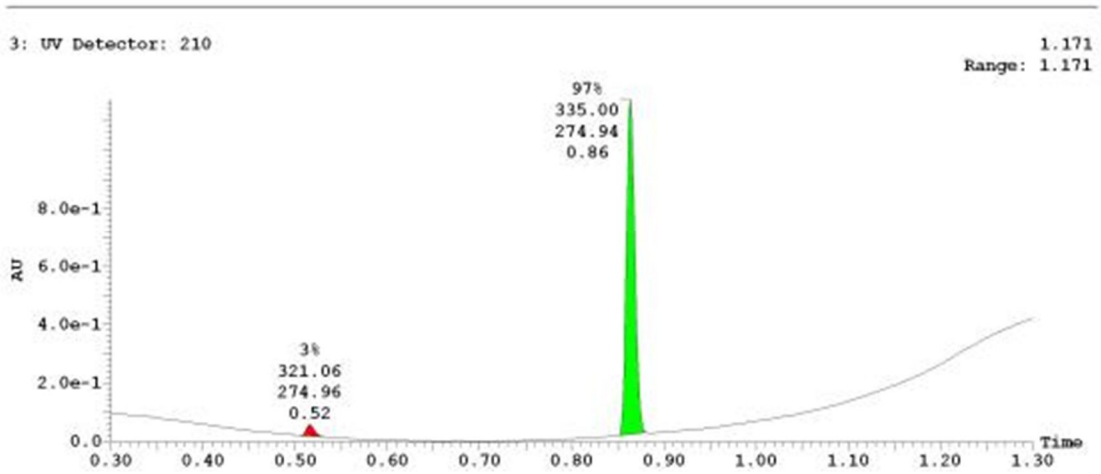
UPLC-MS of Analog CID 46856253



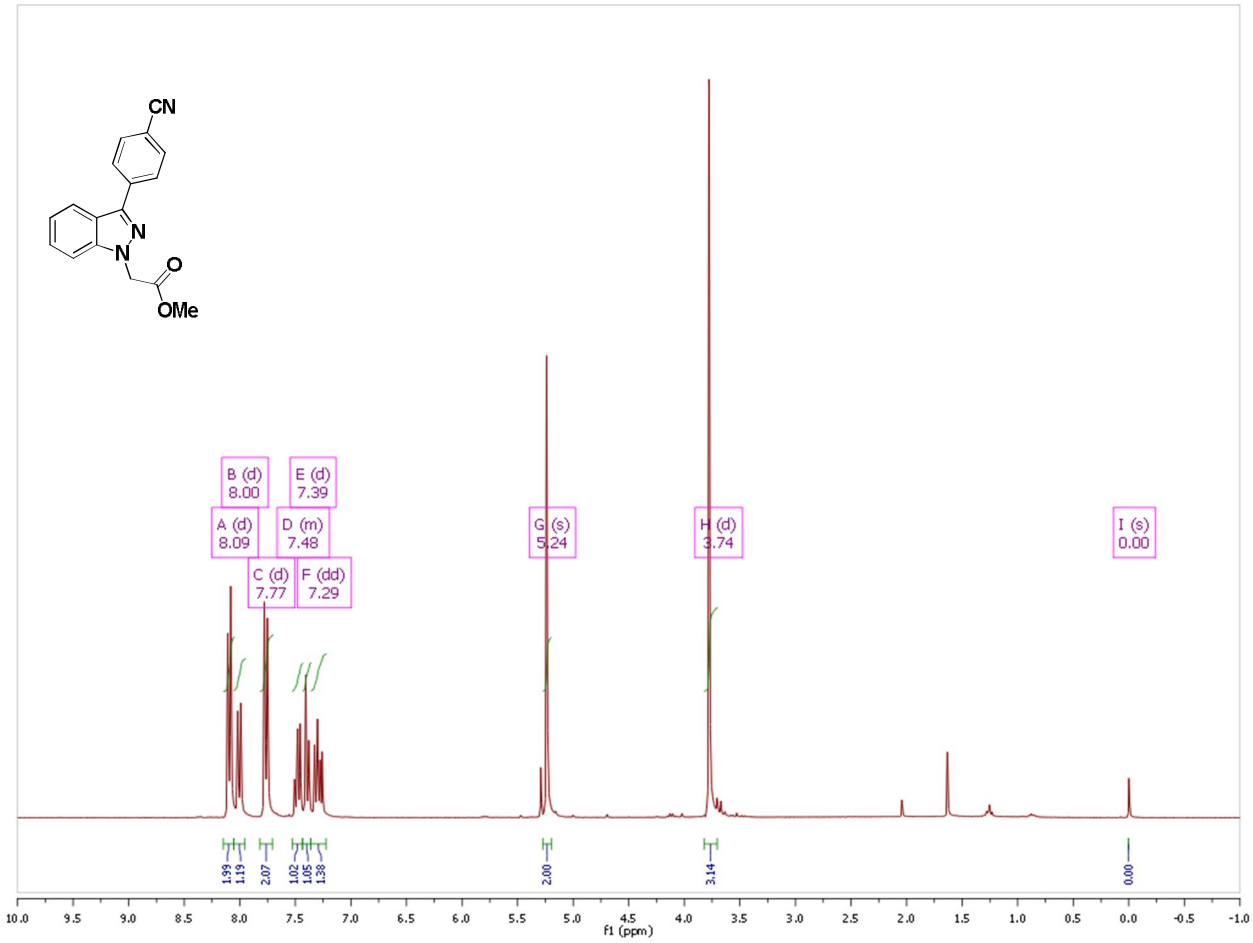
^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 49835854 (Table 7, entry 5)



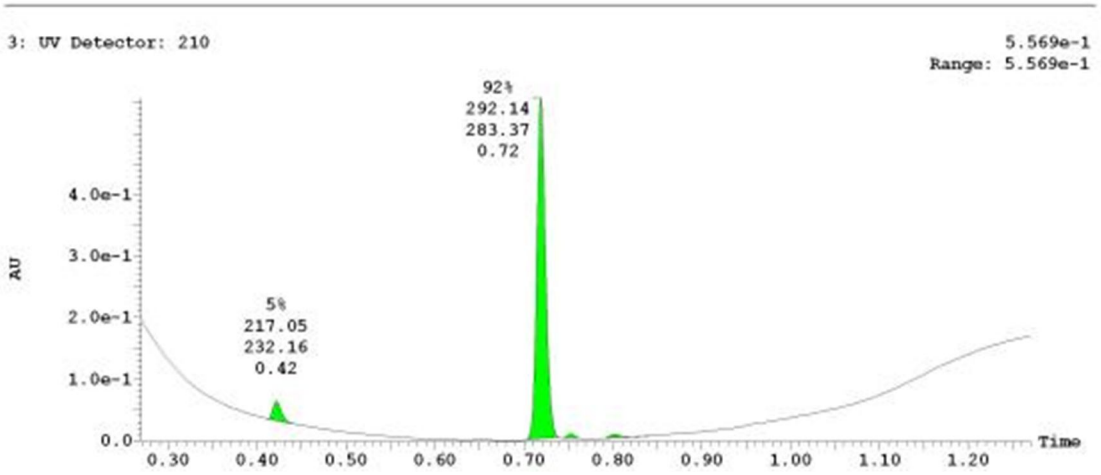
UPLC-MS of Analog CID 49835854



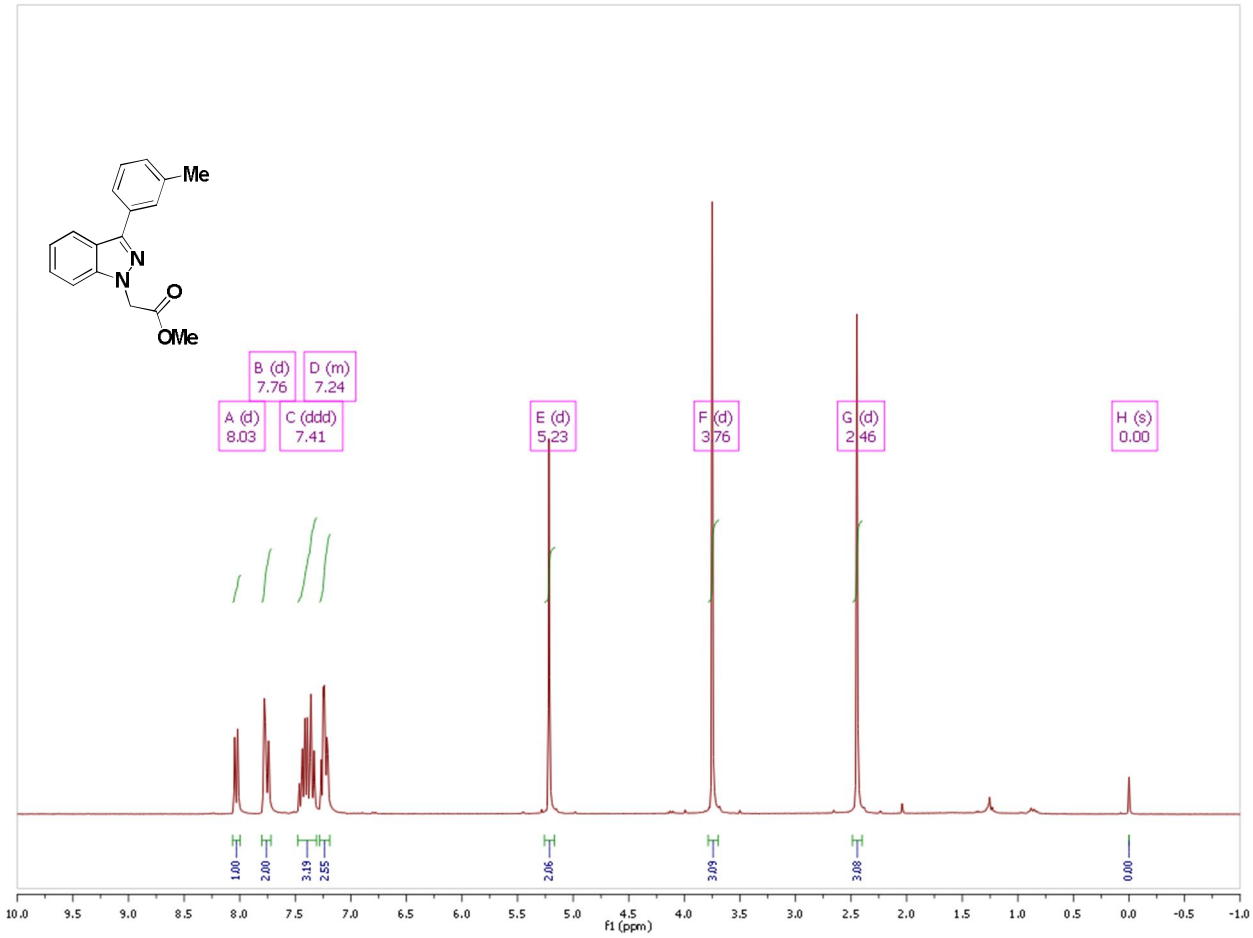
^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 49835863 (Table 7, entry 6)



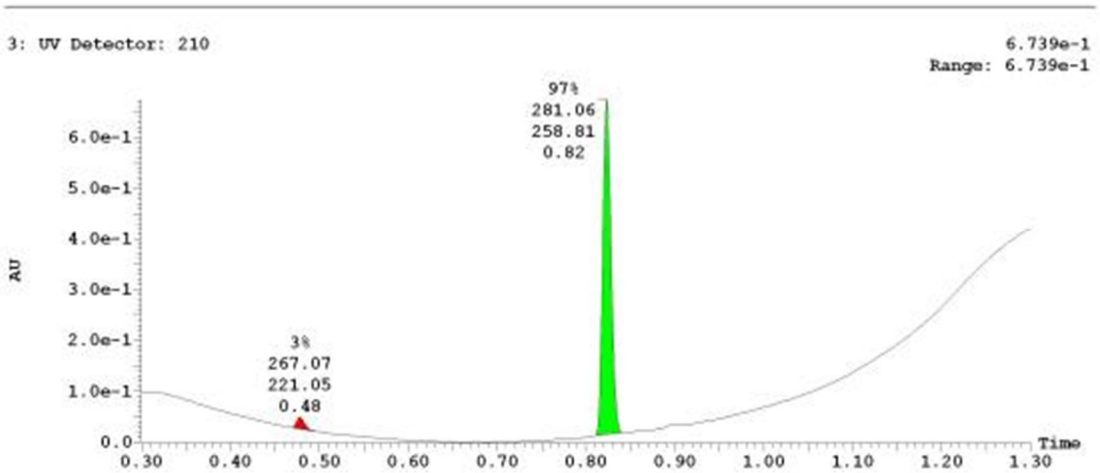
UPLC-MS of Analog CID 49835863



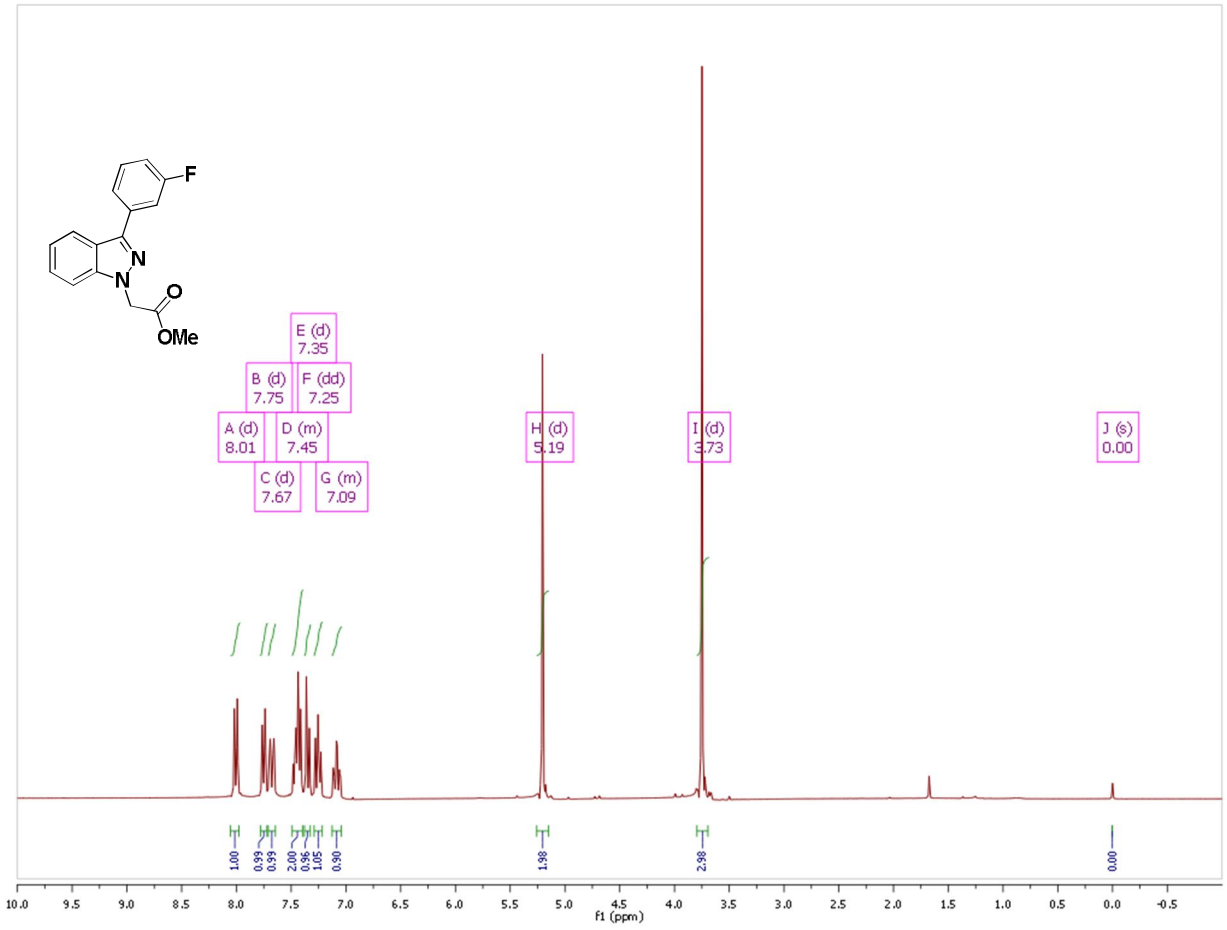
^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 49835823 (Table 7, entry 7)



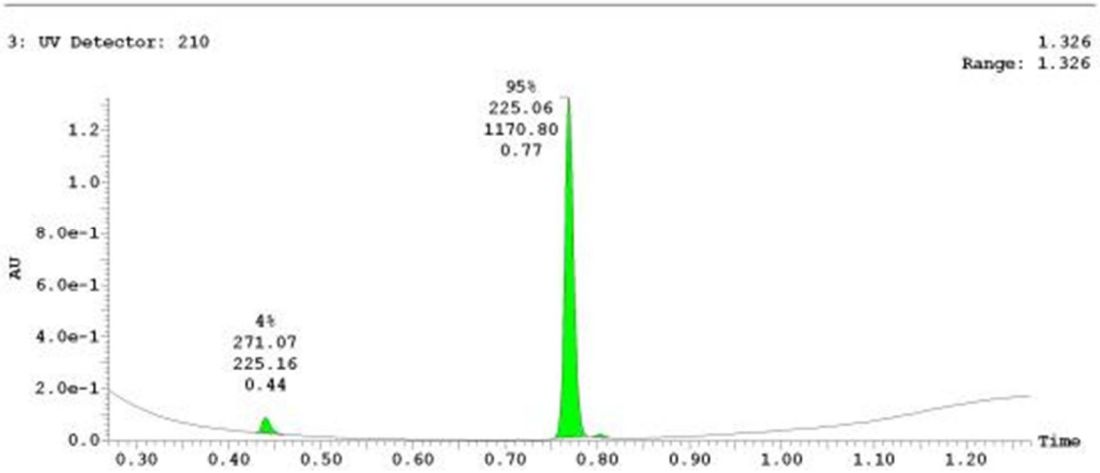
UPLC-MS of Analog CID 49835823



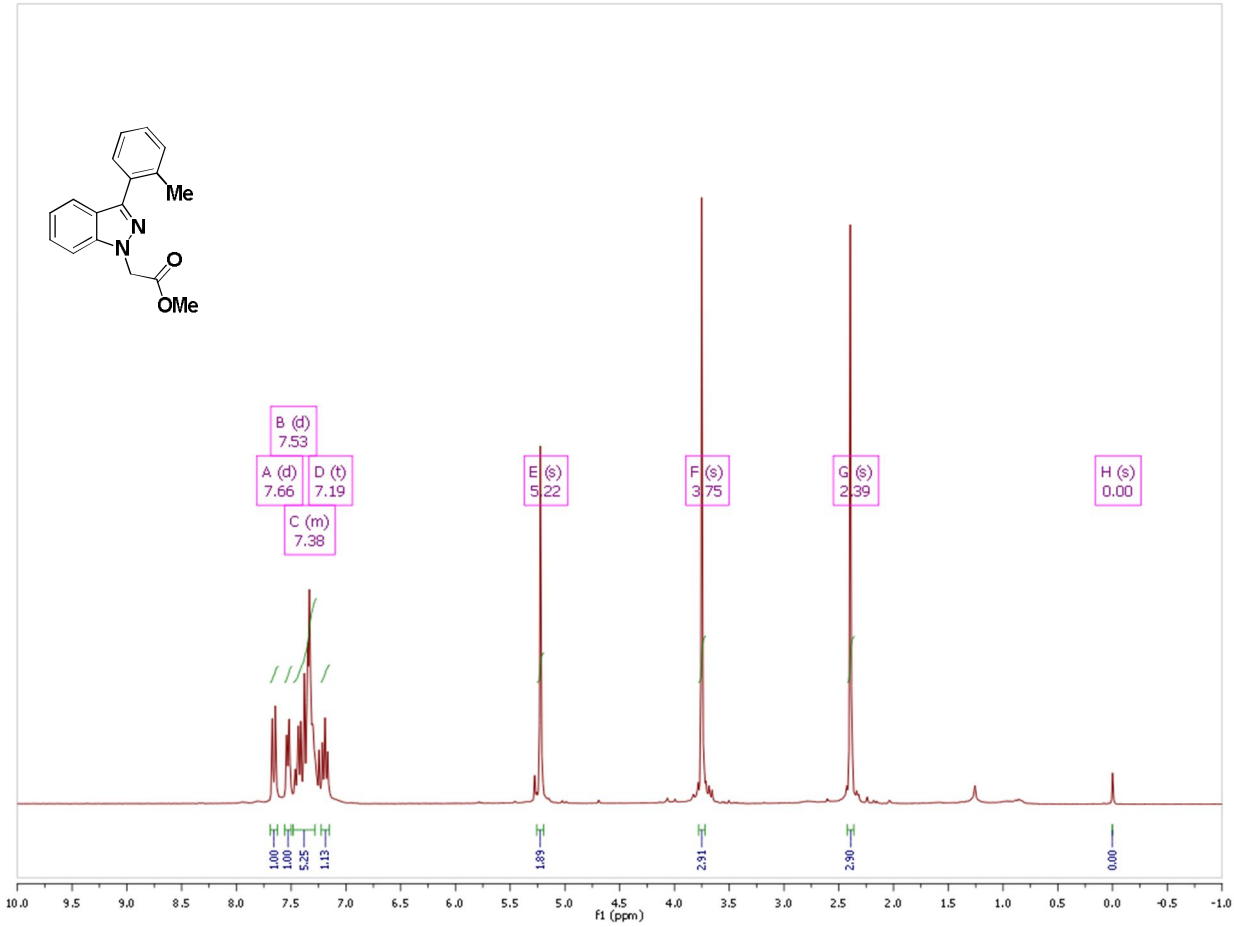
^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 49835858 (Table 7, entry 9)



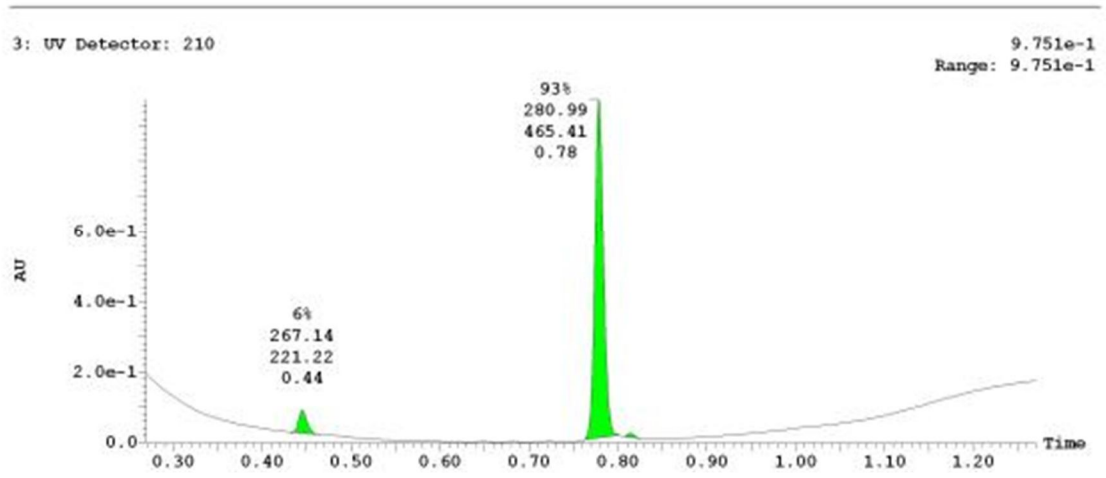
UPLC-MS of Analog CID 49835858



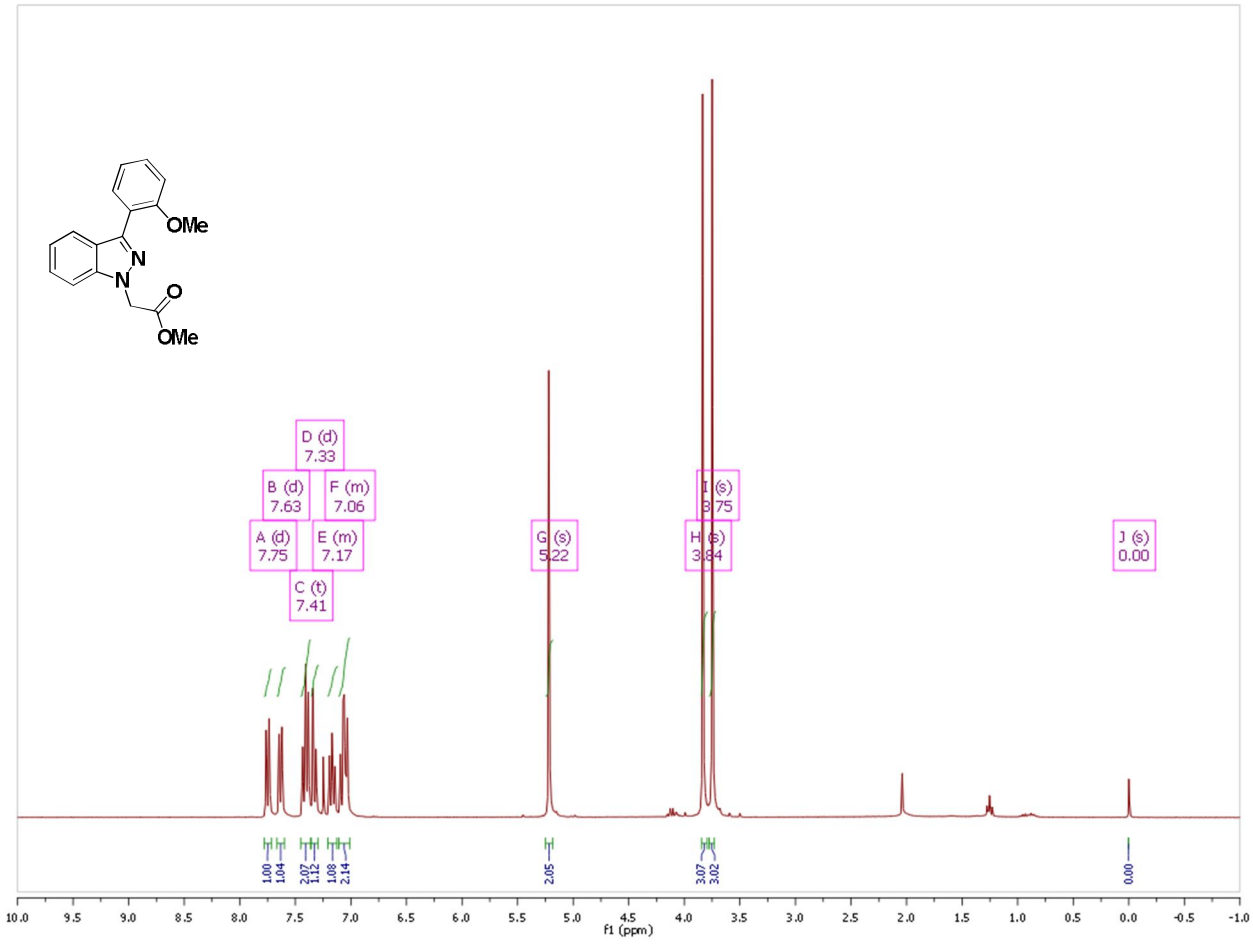
^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 49835865 (Table 7, entry 10)



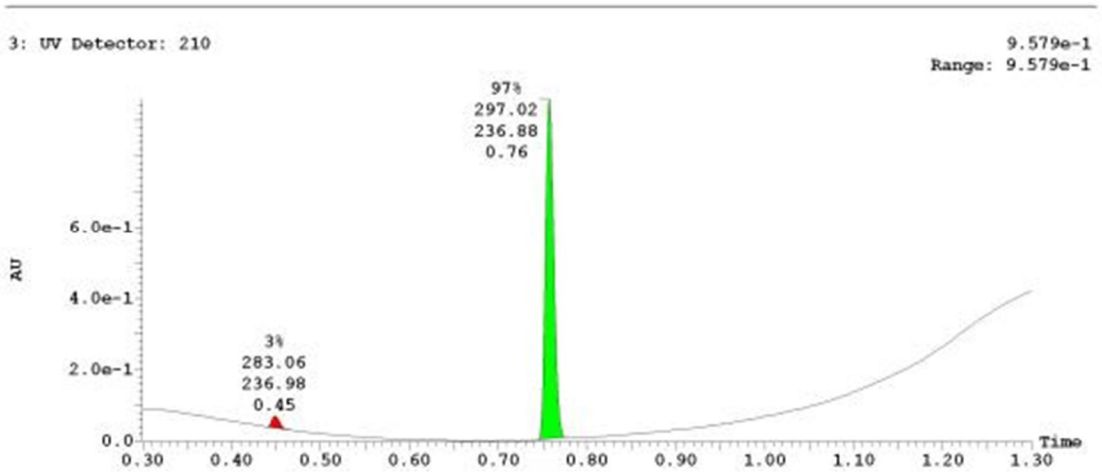
UPLC-MS of Analog CID 49835865



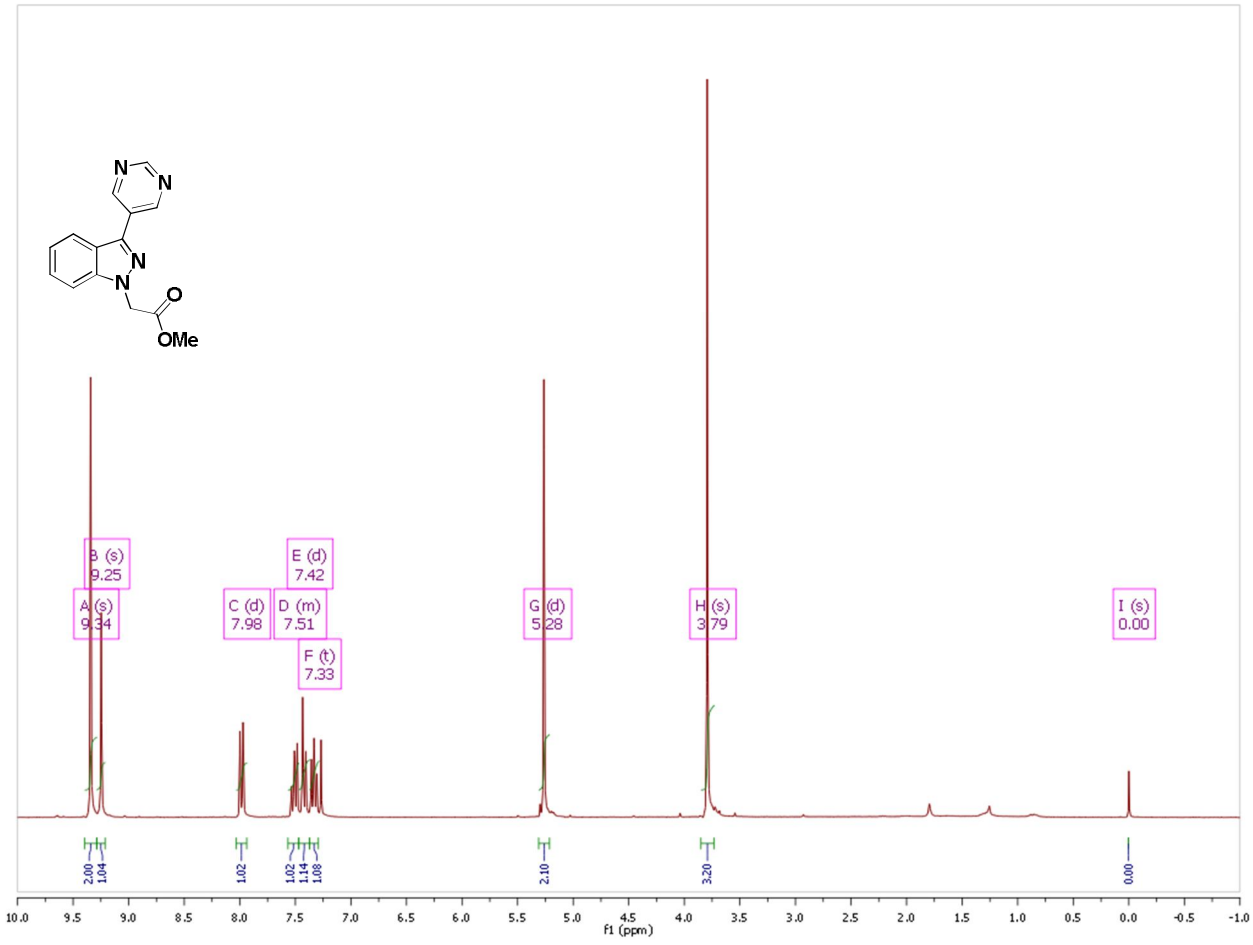
^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 49835850 (Table 7, entry 11)



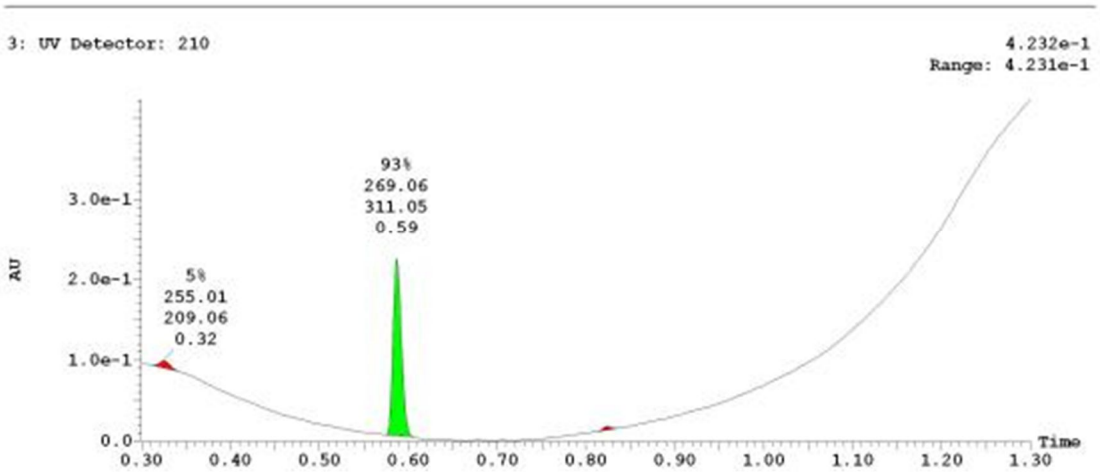
UPLC-MS of Analog CID 49835850



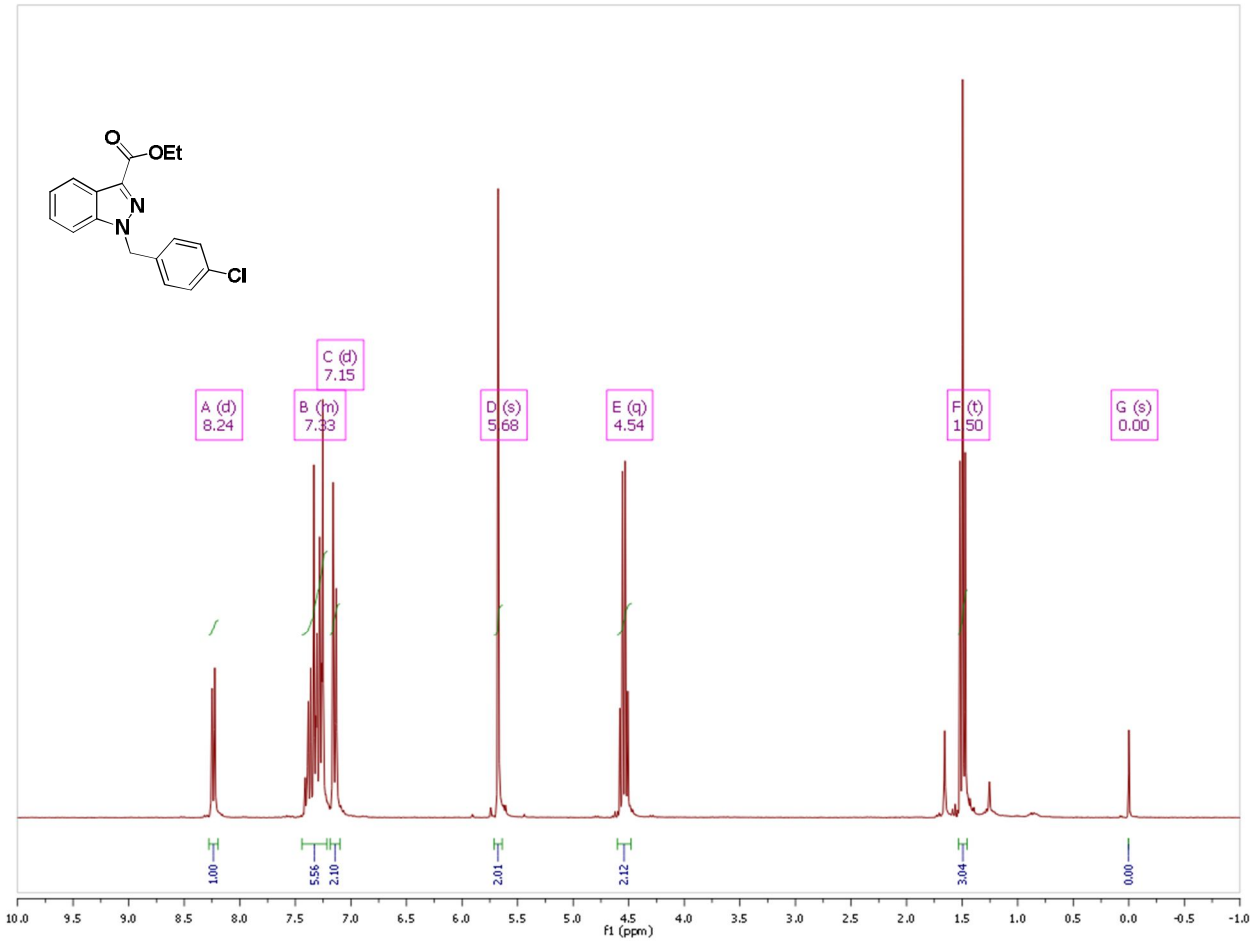
^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 49835868 (Table 7, entry 12)



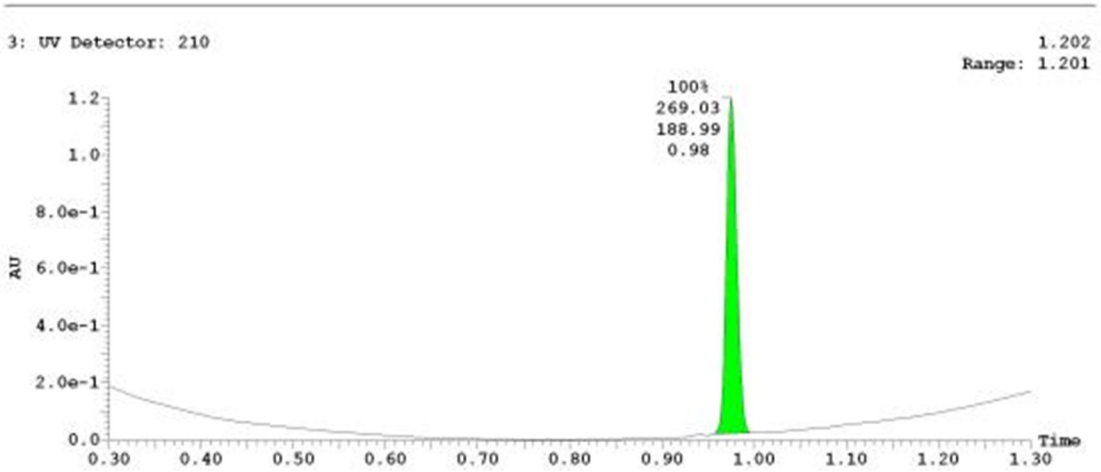
UPLC-MS of Analog CID 49835868



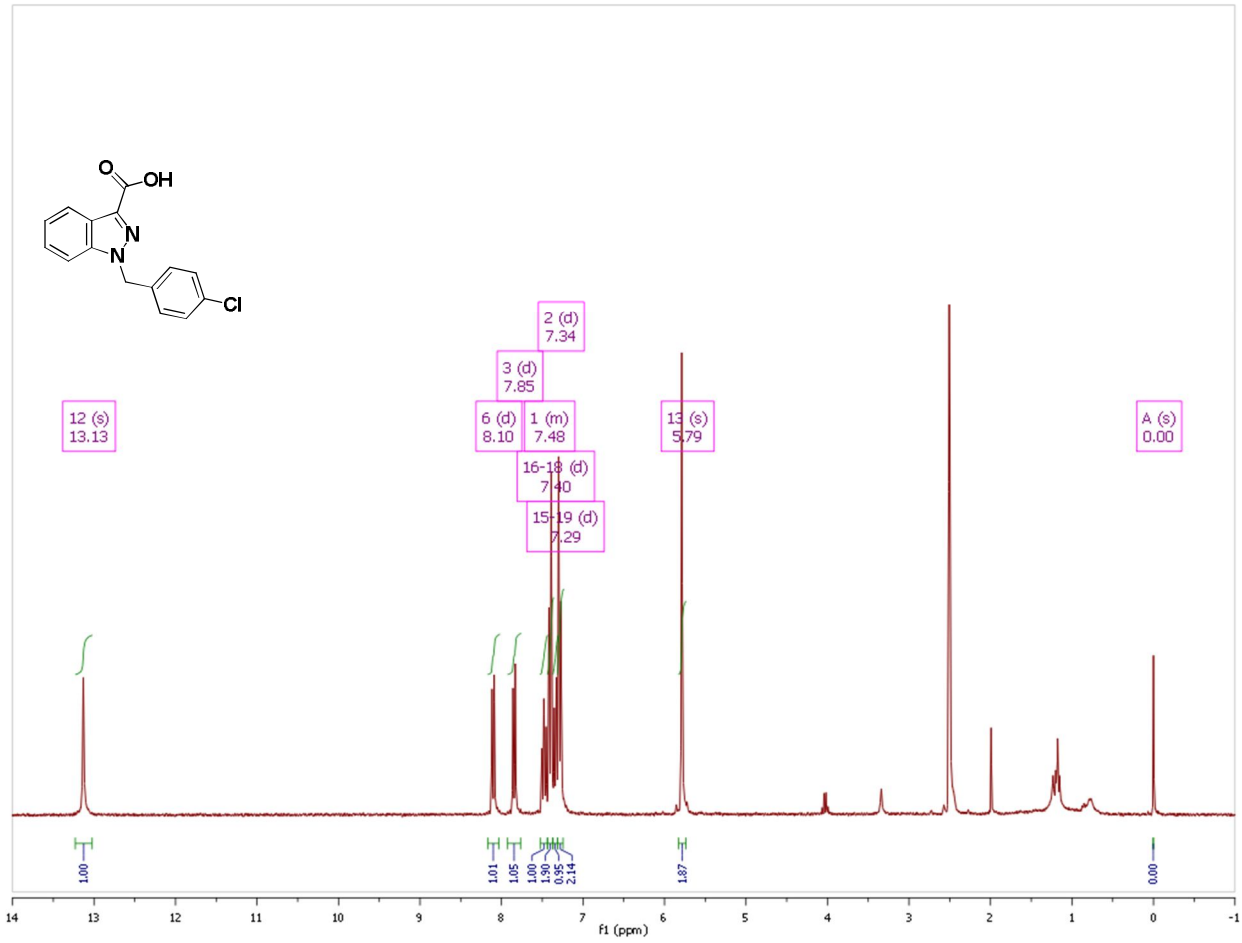
^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 12312246 (Table 8, entry 1)



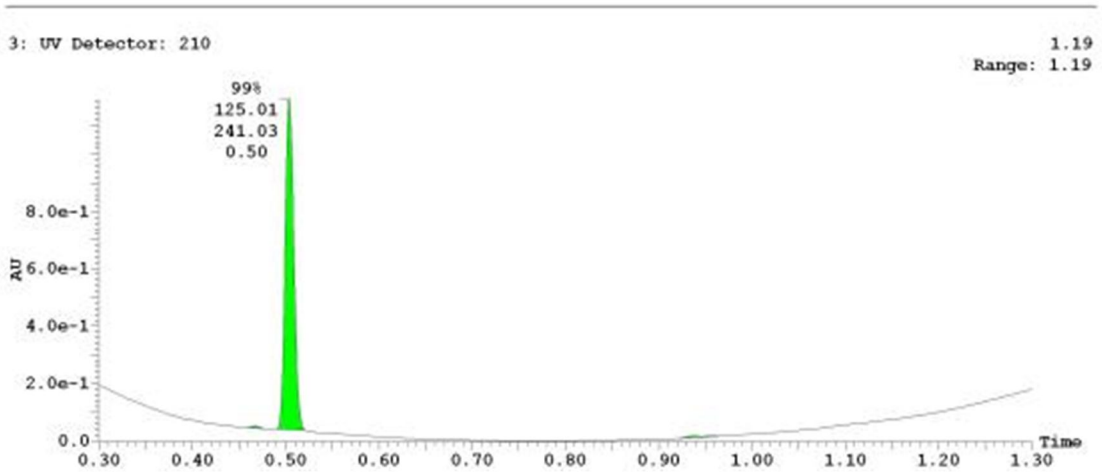
UPLC-MS of Analog CID 12312246



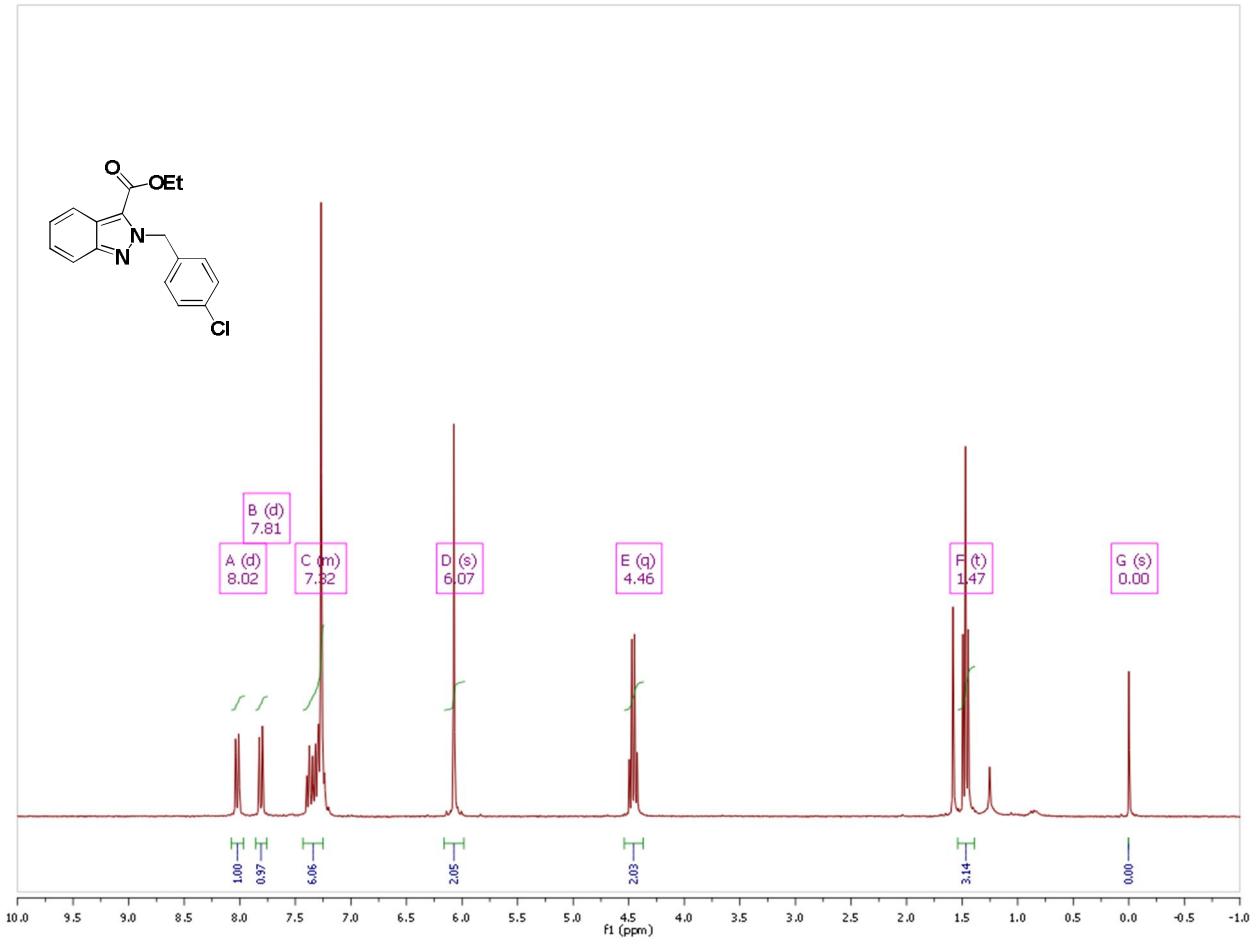
^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 100493 (Table 8, entry 2)



UPLC-MS of Analog CID 100493



^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 46856252 (Table 8, entry 3)



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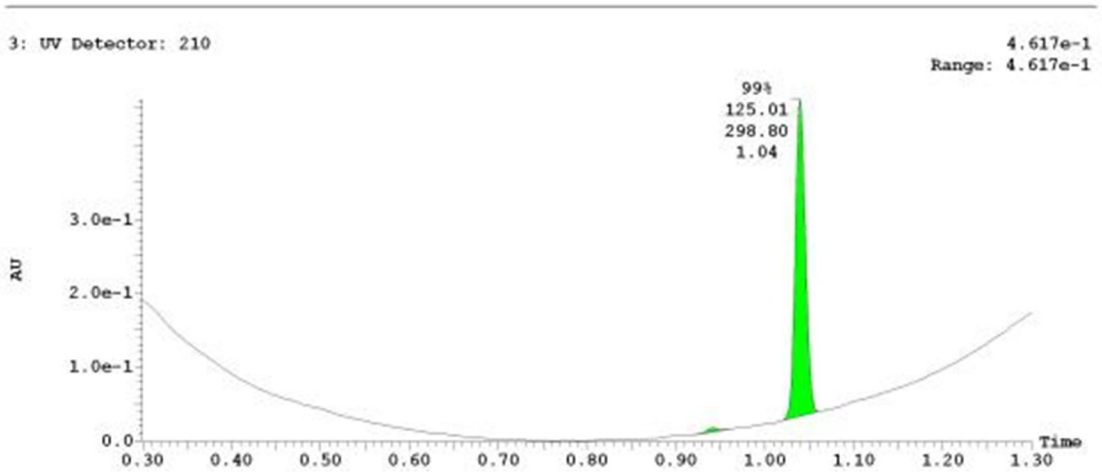
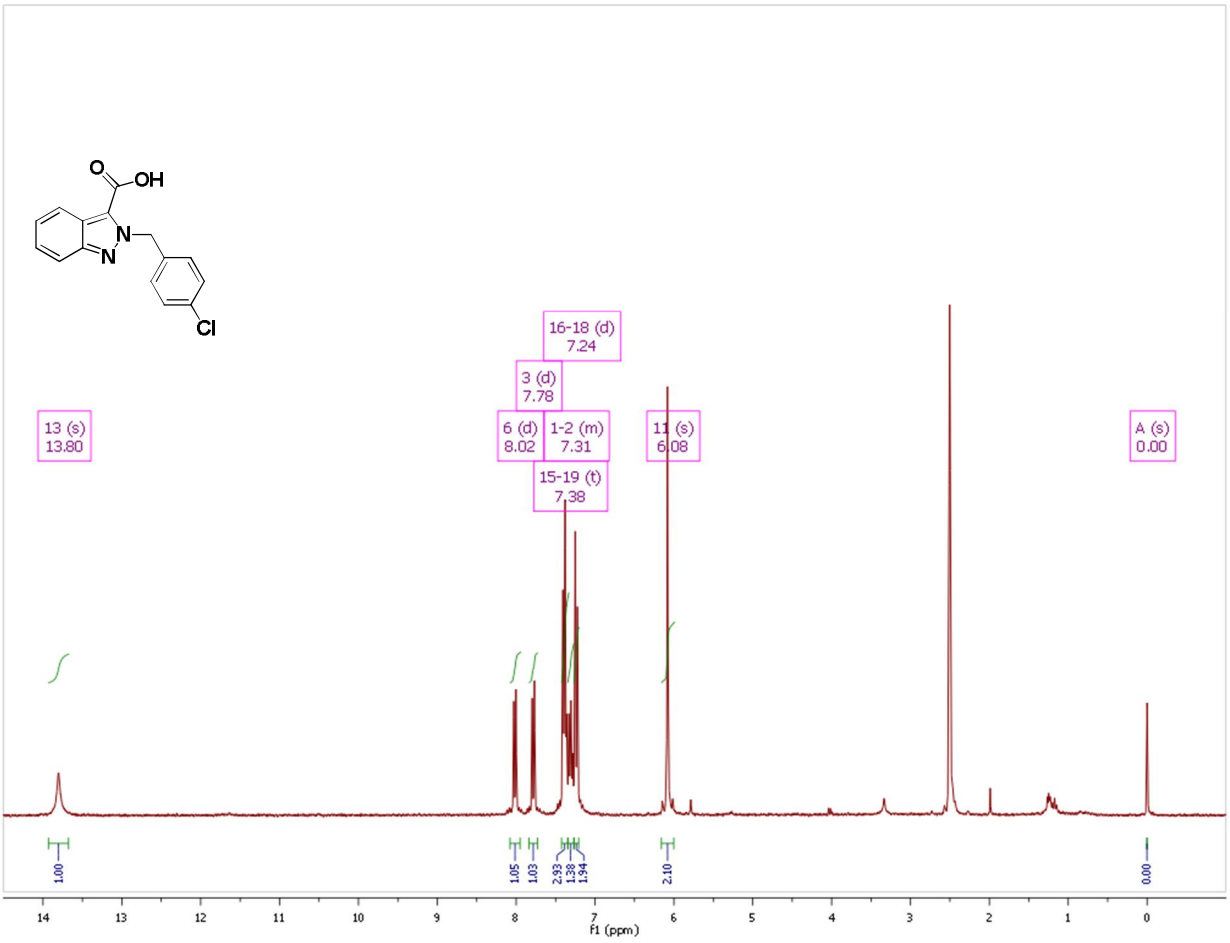
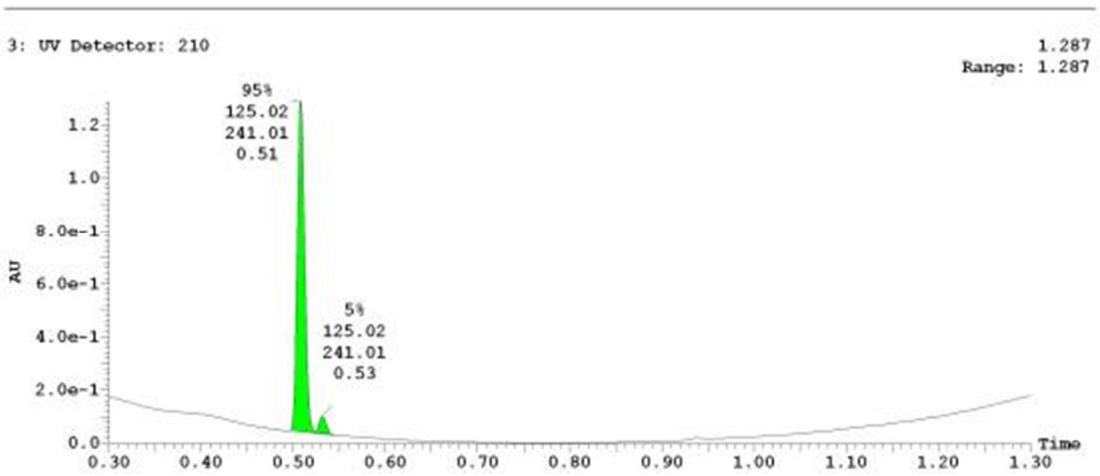


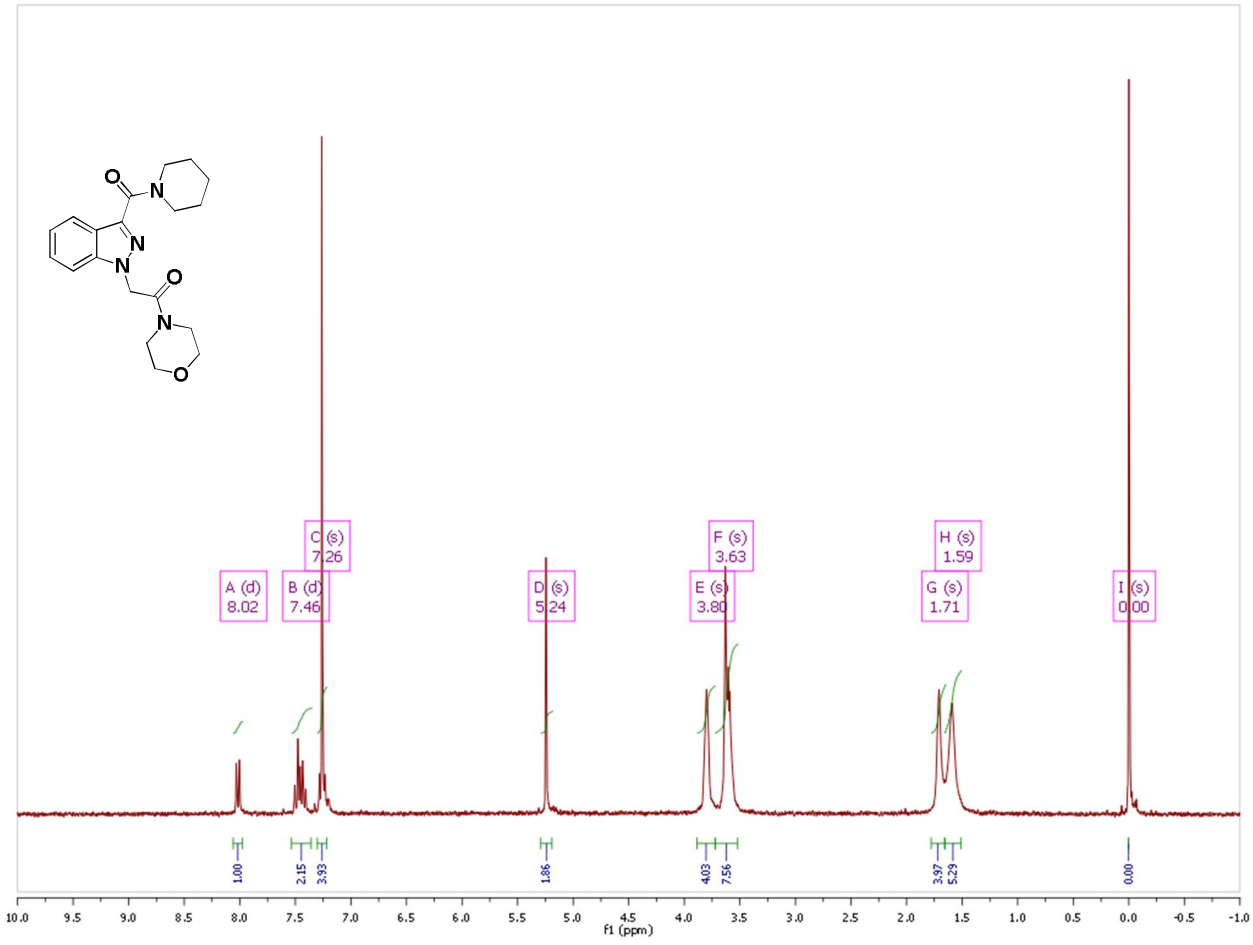
^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 14420692 (Table 8, entry 4)



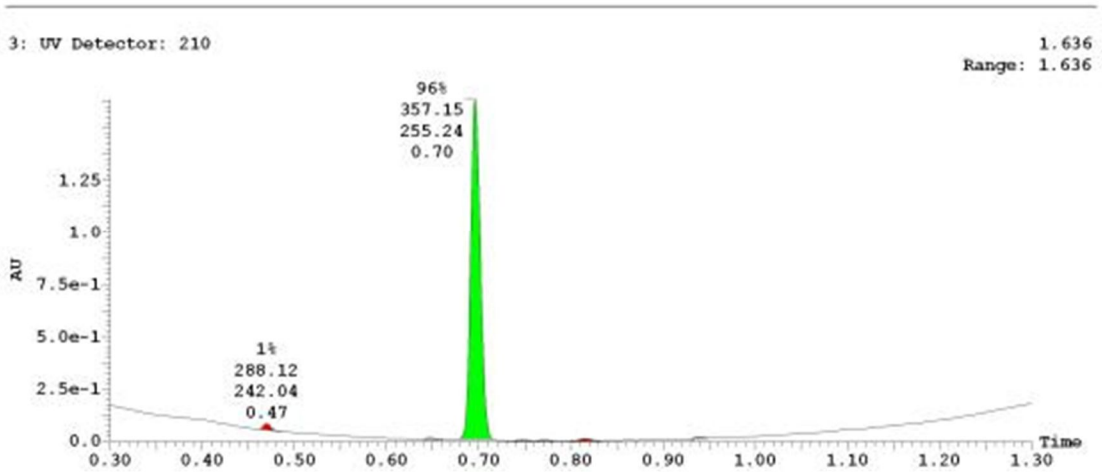
UPLC-MS of Analog CID 14420692



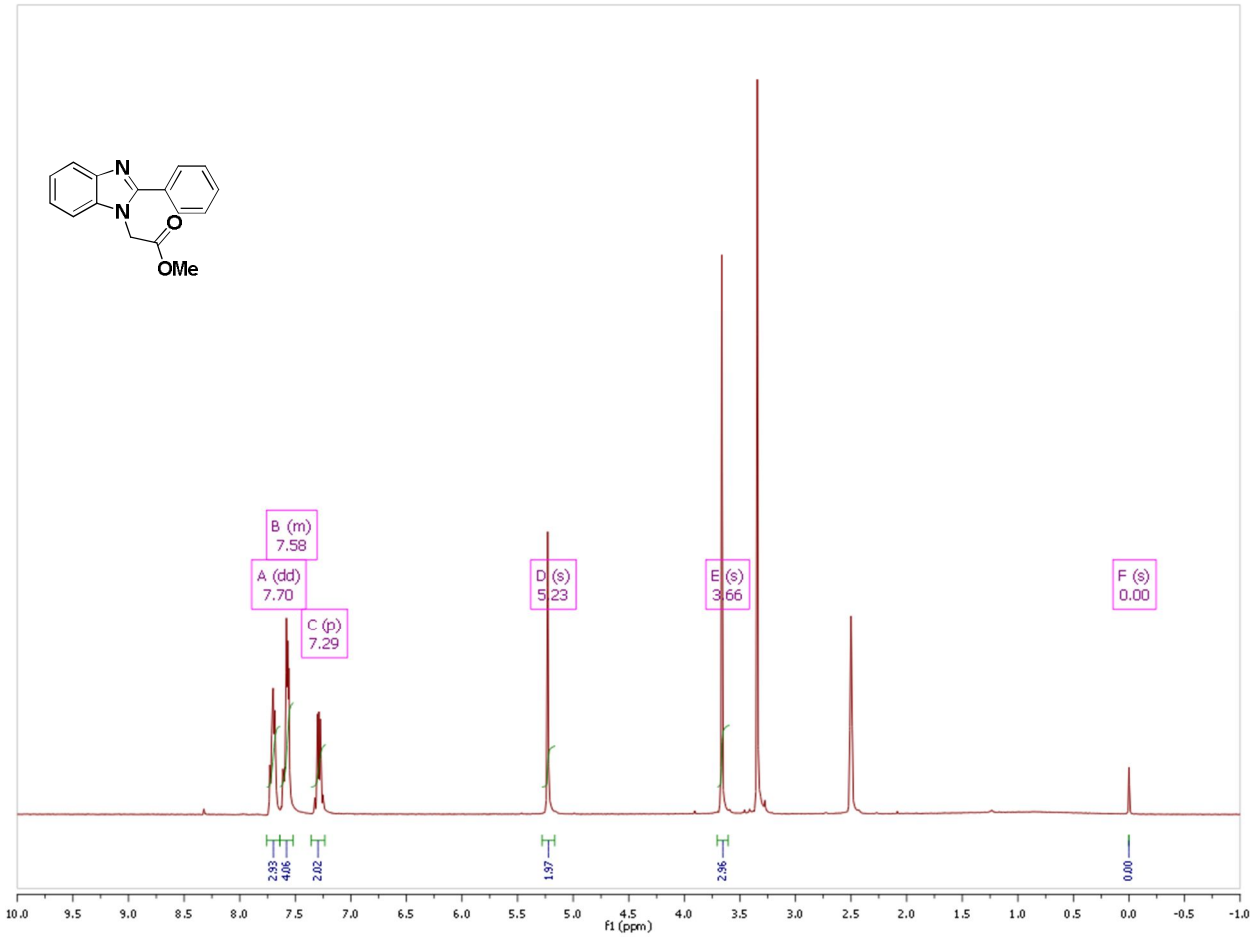
^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 46378661 (Table 8, entry 5)



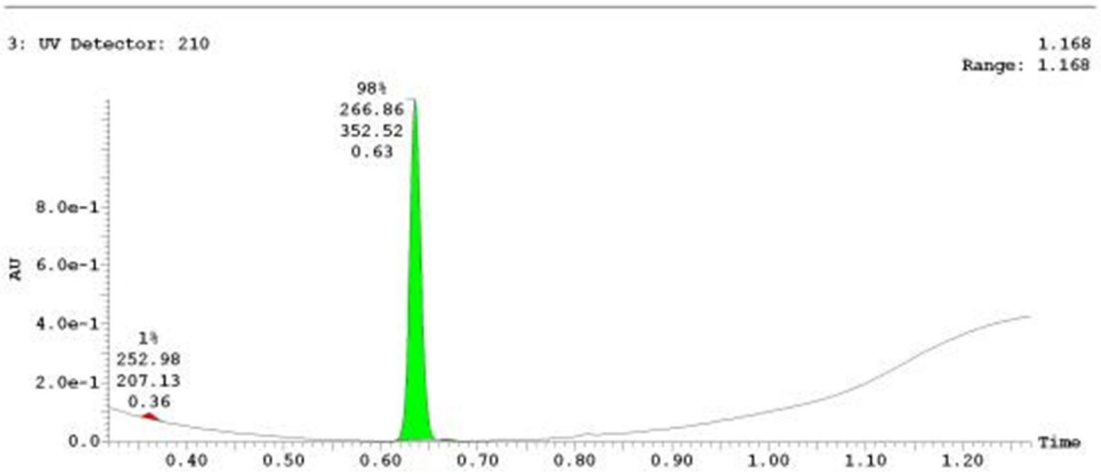
UPLC-MS of Analog CID 46378661



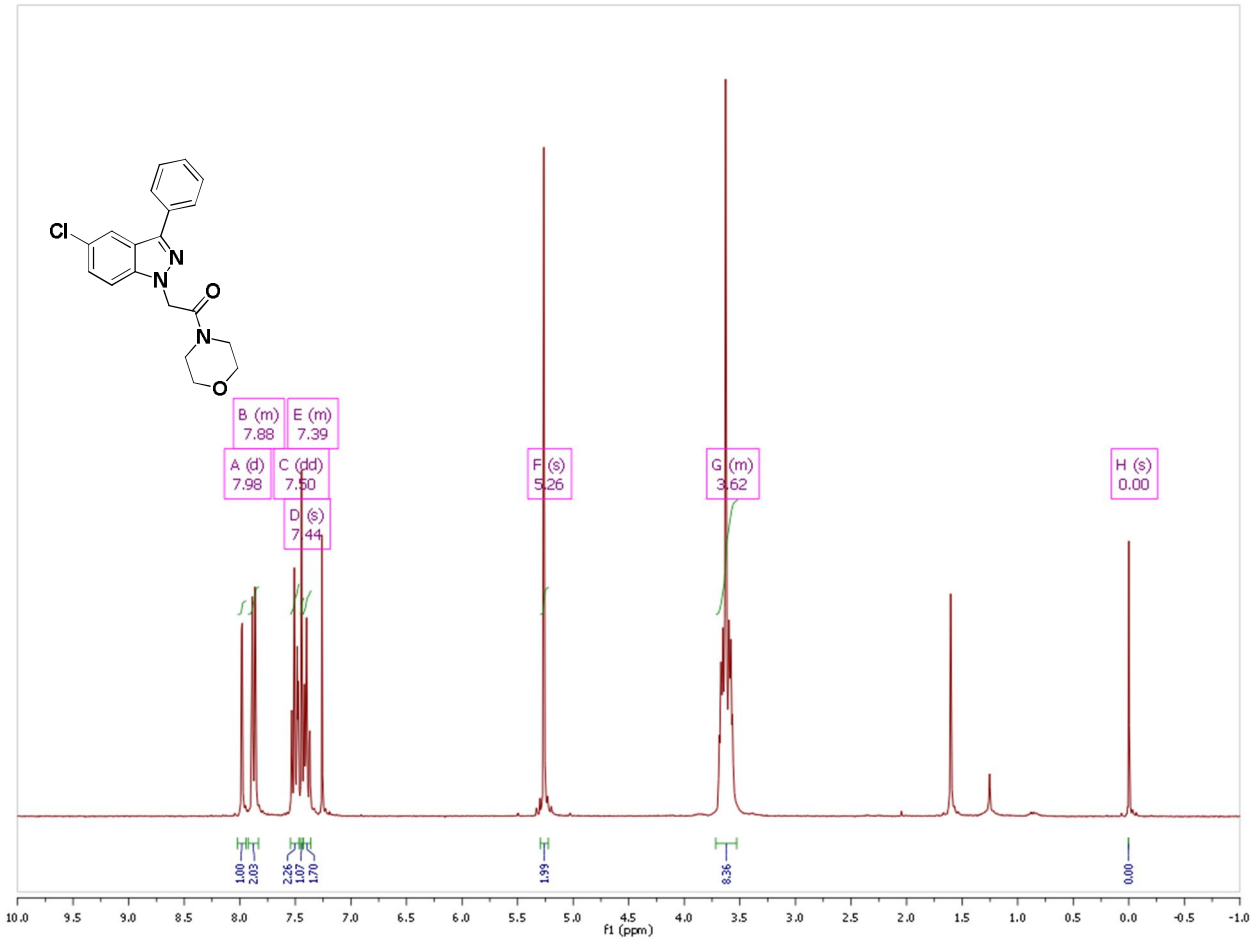
^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 4131200 (Table 8, entry 6)



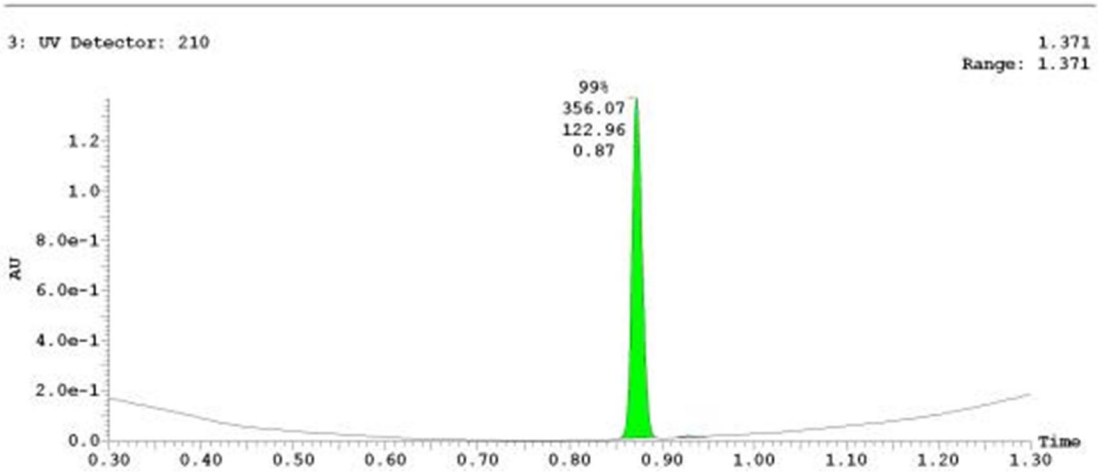
UPLC-MS of Analog CID 4131200



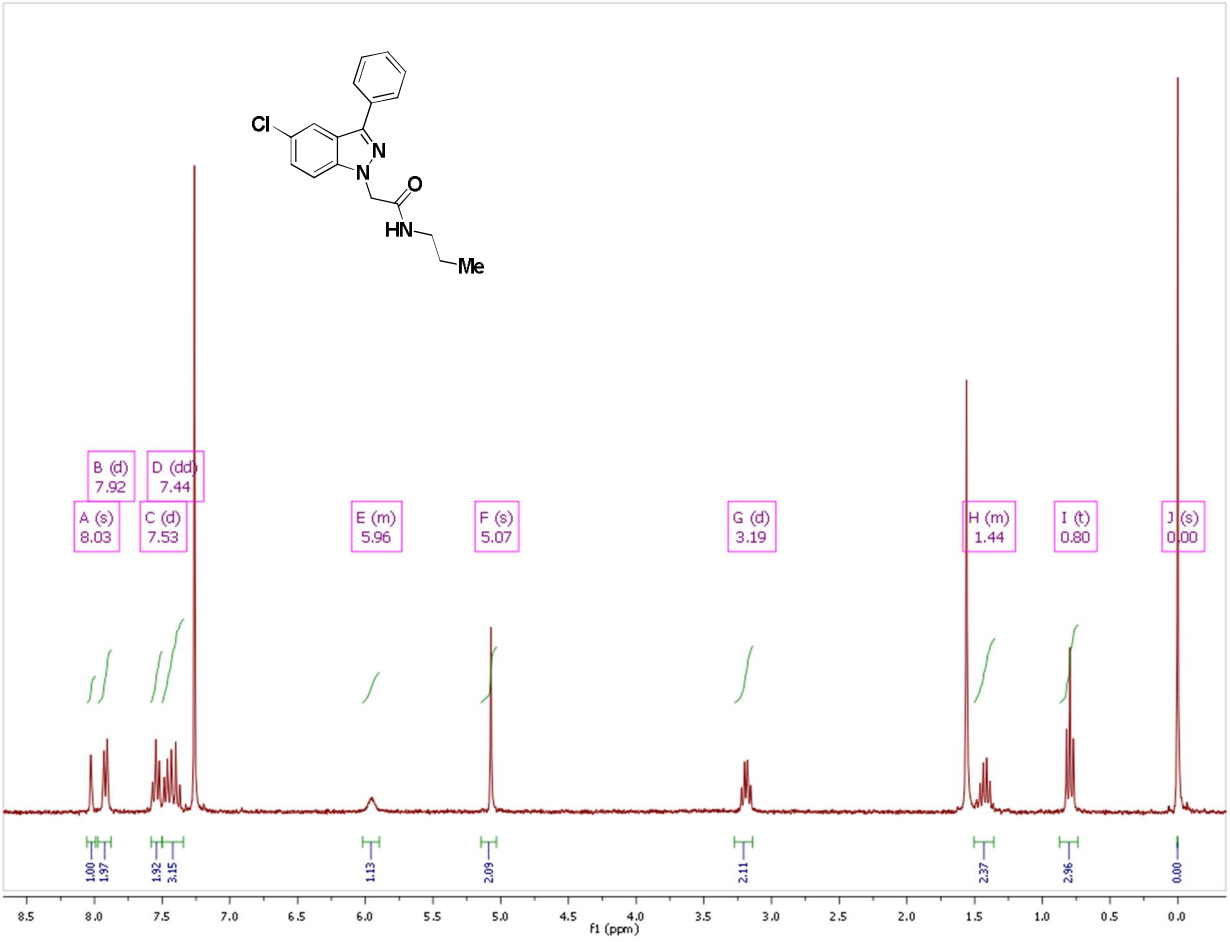
^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 20877381 (Table 8, entry 7)



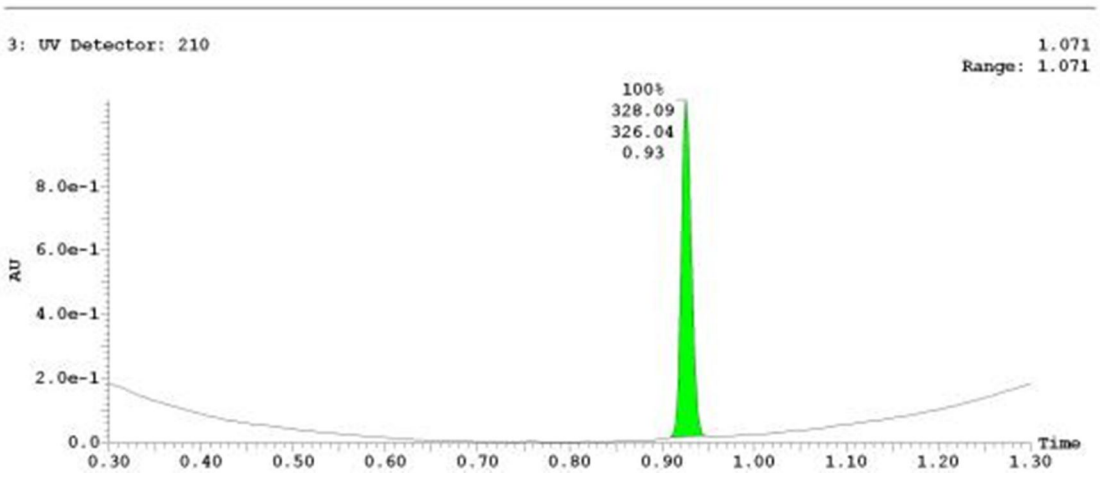
UPLC-MS of Analog CID 20877381



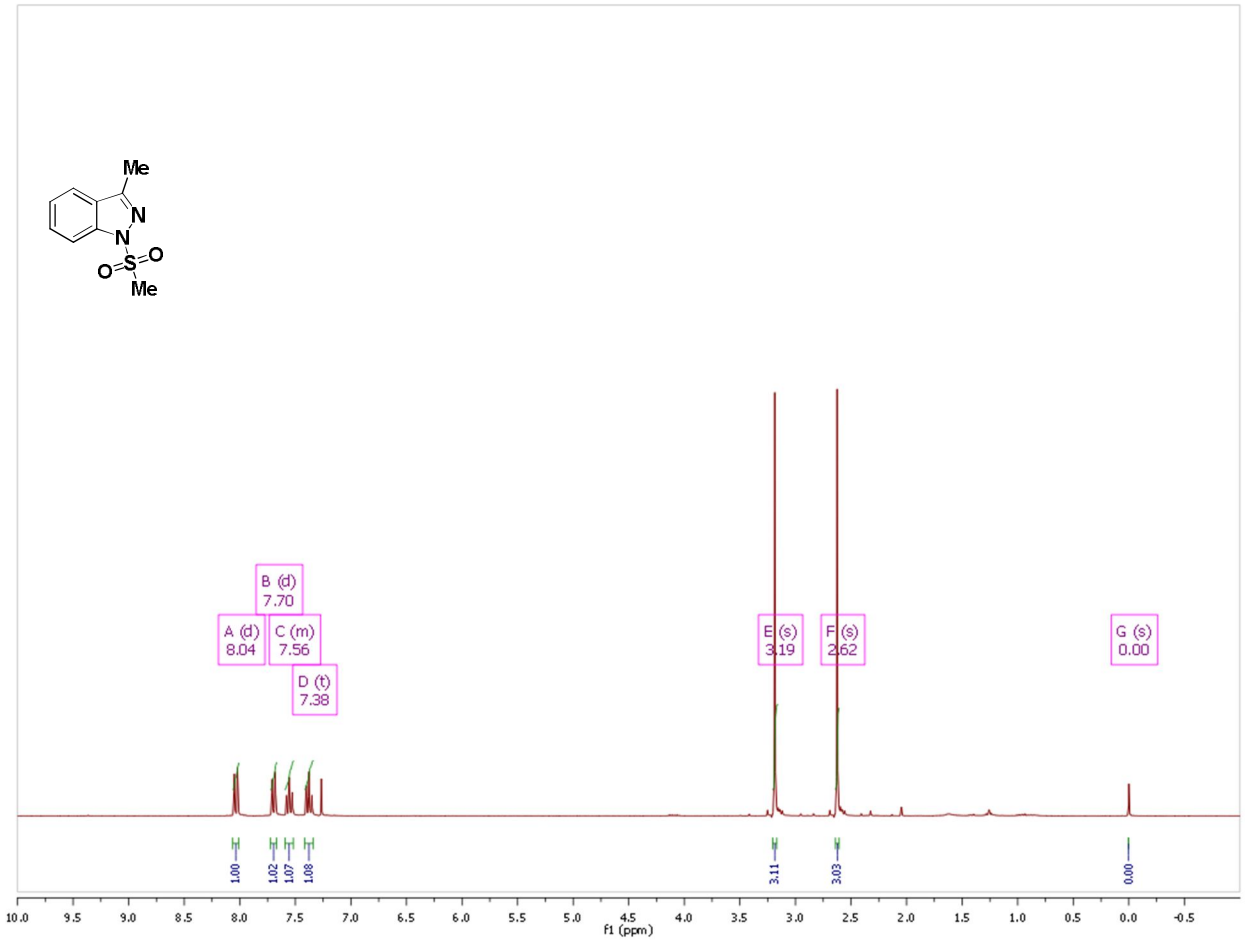
^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 20877322 (Table 8, entry 8)



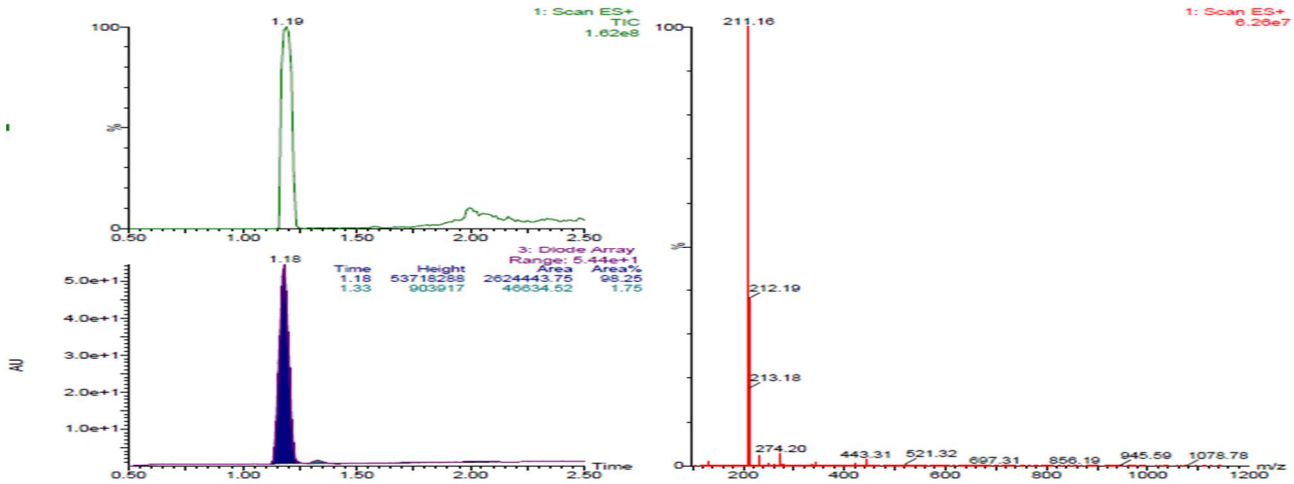
UPLC-MS of Analog CID 20877322



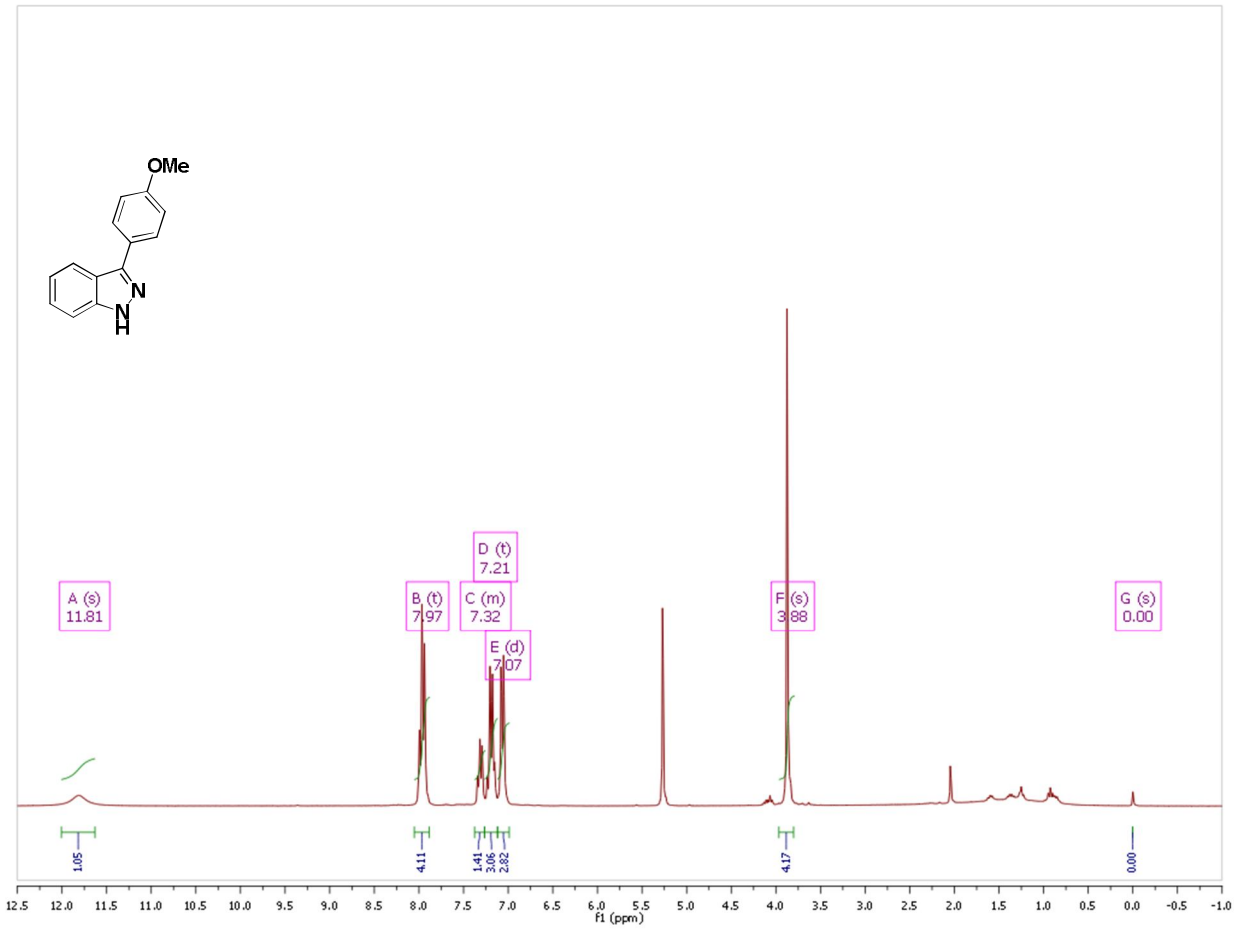
^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 46856251 (Table 8, entry 9)



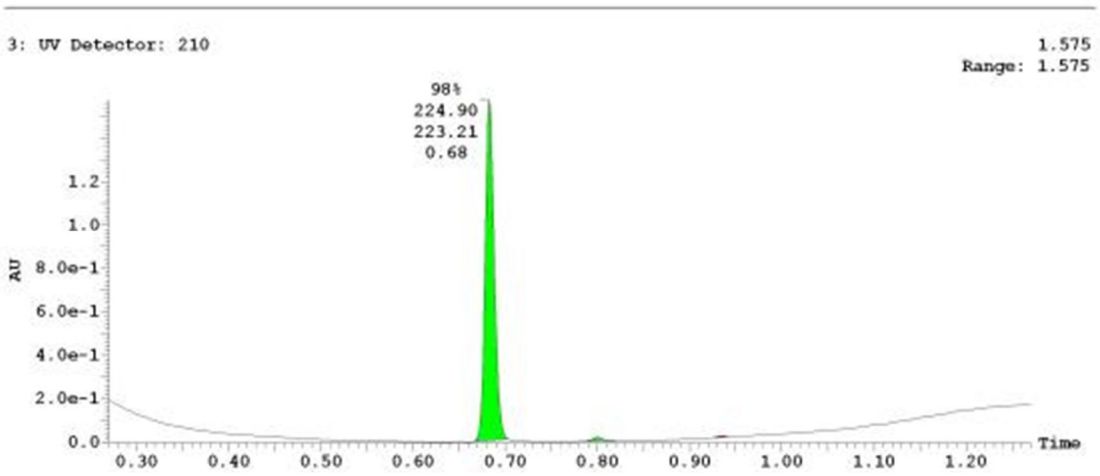
LC-MS of Analog CID 46856251



^{1}H NMR Spectra (300 MHz, $CDCl_3$) of Analog CID 9855970 (Table 8, entry 10)



UPLC-MS of Analog CID 9855970



Appendix D: Compounds Submitted to BioFocus

Table A2. Probe and Analog Information

BRD	SID	CID	P/A	MLSID	ML
BRD-K14324645-001-02-0	104179792	49835877	Probe	003271341	212
BRD-K32112425-001-02-3	113635283	49835842	Analog	003271344	NA
BRD-K37150847-001-07-7	113635284	3243873	Analog	003271343	NA
BRD-K67484673-001-02-3	113635285	100493	Analog	003271339	NA
BRD-K71142328-001-02-0	113635286	49835836	Analog	003271340	NA
BRD-K98546361-001-02-9	113635287	49835857	Analog	003271342	NA

A = analog; P = probe